THESIS FOR THE DEGREE *CANDIDATA PHARMACIAE*

SYNAPSIN I/II DOUBLE KNOCKOUT MICE AND SPONTANEOUSLY HYPERTENSIVE RATS;

TWO ANIMAL MODELS WITH IMPLICATIONS FOR NEUROLOGICAL DISORDERS

by

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CONTENTS

SUMMARY

The synapsins are abundant phosphoproteins associated with synaptic vesicles. Although synapsins were among the first vesicle proteins discovered, their functions still remain incompletely understood. Previous studies have reported major decreases in the number of synaptic vesicles in synapsin deficient brains, but knowledge about which classes of vesicles are affected has been lacking. In the first part of this study, the consequences of the deletion of synapsin I and II for different subgroups of vesicles were studied in different brain regions.

It was recently reported by our group that the levels of VGLUT1, VGLUT2 and VGAT are decreased by approximately 40% in mice devoid of synapsin I and II. In this study, the main focus was on the effects on cholinergic vesicles, using VAChT as a cholinergic marker, in mice lacking synapsin I and II. The levels of different vesicular transporters were studied in three brain areas, the neostriatum, cortex and pons medulla. The levels of VGLUT-1, VGLUT-2 and VGAT were decreased by 30-50% in all the brain areas examined. In contrast, the levels of VAChT were decreased by only 23% in the neostriatum, and were present at the same levels as wild-type in cortex and pons medulla. We do not know whether the cholinergic terminals in striatum, cortex and pons medulla, differ regarding their dependence and co/localization with synapsins. Synaptic vesicles are covered with synapsins and could serve some role of vesicle stabilization. A decrease in synaptic vesicles in mice lacking synapsins could indicate that synapsins serve a role in vesicle stabilization. Lack of synapsins could therefore induce the vesicles to undergo spontaneous degradation..

In the second part of the study, the aim was to measure the amounts of proteins involved in the dopaminergic system in an animal model for the “attention deficity hyperactivity disorder” (ADHD). The spontaneous hypertensive rat (SHR) is the only animal model that has been found to demonstrate all the behavioural characteristics of ADHD, namely hyperactivity, impulsivity and problems with sustained attention. Since abnormal dopaminergic responses appear as one of main markers for ADHD, the amount of dopamine D_1–like receptors was measured. Our results show an increase by 21% in the density of D_1-like receptors in cerebrum. This increase in the amounts of D1-like receptors could be a compensatory mechanism for dopamine hypofunction (evt reduced dopamine

release) in SHR, and confirms the hypothesis of a dysregulation of the dopaminergic system in ADHD. No difference was found in the levels of the D_1-interacting protein, calcyon, or the enzyme which catalyzes the degradation of dopamine, COMT. Since many dopaminergic effects are mediated through interaction with glutamatergic neurotransmission, the levels of the NMDA-subunits of the NMDA-receptor were examined in neostriatum and cerebrum. No changes were detected on the protein level of NMDA-1 and NMDA-2A/B, but one cannot exclude their involvement in the aetiology of ADHD.

ABBREVIATIONS

aa	Amino acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADHD	" Attention-deficit hyperactivity disorder"
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumine
CaM	Ca^{2+}/calmodulin dependent protein kinase I
cAMP	cyclic Adenosine Mono Phosphat
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
DKO	Double Knockout
ES cells	embryonic stem cells
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
HRP	Horseradish Peroxidase
5-HT	Seretonin
kDa	kilo Dalton
KO	Knockout
NMDA	N-methyl-D-aspartate
PKA	Protein Kinase A
PNS	Periferal Nervous System
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulphat
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel
SHR	Spontaneuosly hypertensive rat
TBS	Tween- Tris-buffered salt solution with tween 20
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine
VAMP	vesicle-associated membrane protein
VGLUT	vesicular glutamate transporter
VGAT	vesicular GABA transporte
VAChT	vesicular acetylcholine transporter
VMAT	vesicular monoamine transporter

w/v	weight/volume
WKY	Wistar-Kyoto
WT	Wild Type

1. INTRODUCTION

1.1 THE NERVOUS SYSTEM

The brain is composed of glia cells and neurons. Every neuron is divided in four structurally different unities: a cell body, dendrittes, axon and thousands of nerveterminals. A single neuron can be connected to many other neurons and the total number of neurons and connections in a network can be extremely large. Connections, called synapses, are usually formed from axon neuroterminals to dendrittes.

1.1.1 SYNAPTIC TRANSMISSION

The connections between neurons are abundant in the brain. These connections, better known as synapses, branch into three groups regarding their physiological typing, namely excitatory, inhibitory and modulatory. Regarding the synaptic type, there is a chemical synapse which enable cell-to-cell communication via secretion of neurotransmitters, or less common, an electrical synapse, where signals are transmitted through gap junction (Cohen-Cory, 2002; Siegel G.J. *et al*., 1999).

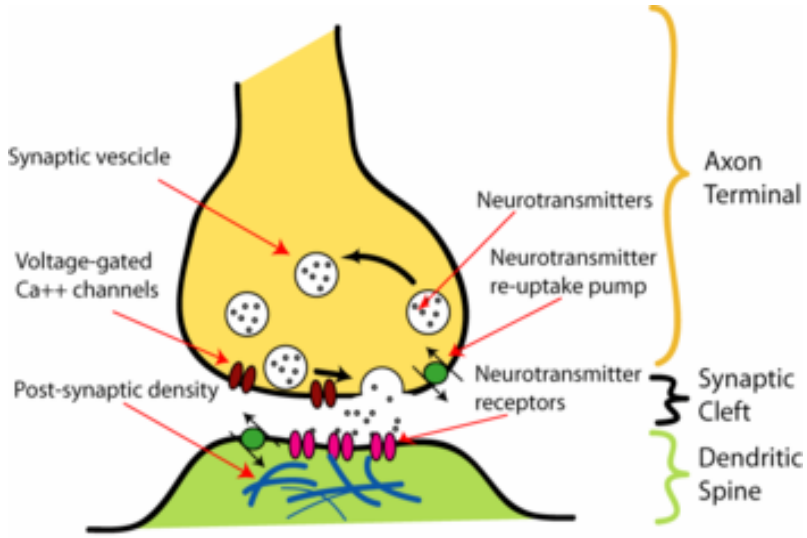


Figure 1.1 An illustration of synaptic transmission

Chemical neurotransmission is the major means by which nerves communicate with one another in the nervous system. Synaptic transmission is initiated when an action potential triggers neurotransmitter release from a presynaptic nerve terminal, as shown in figure 1.1 (Sudhof, 2004). Stimuli caused by an action potential, opens voltage-gated Ca^{2+} channels, triggering exocytose of neurotransmitters from synaptic vesicles. Released

neurotransmitters interact with receptors in the postsynaptic membrane, thereby activating it. Released neurotransmitters are inactivated either by reuptake into the nerve terminal by neurotransmitter re-uptake pump, by degradation or by uptake and metabolism by glial cells (Masson *et al.*, 1999).

1.1.2 THE SYNAPTIC VESICLE CYCLE

The presynaptic nerve terminal is filled with small translucent synaptic vesicles. These organelles are abundant, and are approximately ~20 nm in radius (Sudhof, 2004).The major function of synaptic vesicles is to take up and store neurotransmitters, and to fuse and bud from membranes.

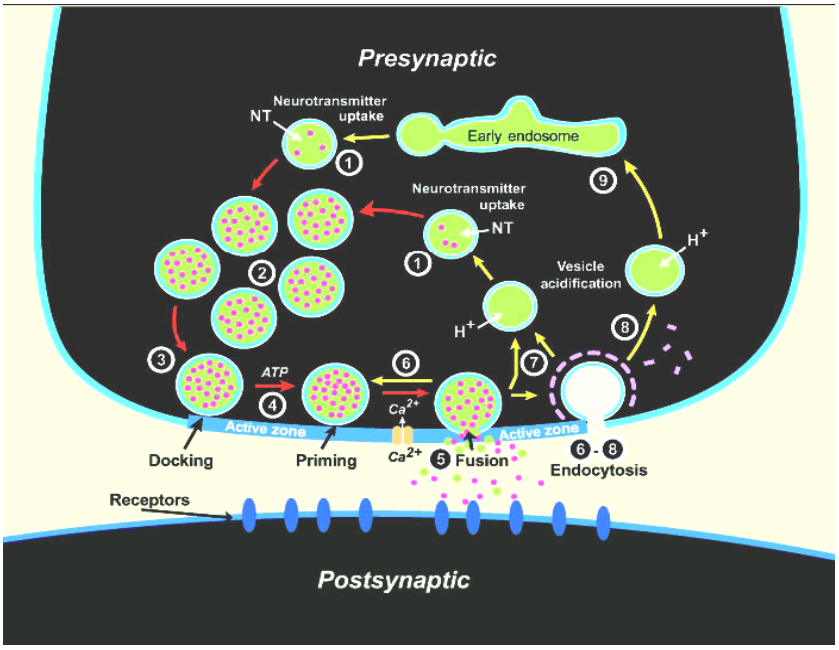


Figure 1.2 The synaptic vesicle cycle: Synaptic vesicles are filled with neurotransmitters by active transport (step 1) and form the vesicle cluster that may represent the reserve pool (step 2). Filled vesicles dock at the active zone (step 3), where they undergo a priming reaction (step 4) that makes them competent for Ca^{2+} triggered fusion-pore opening (step 5). After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle via several routes: local reuse (step 6), fast recycling without an endosomal intermediate (step 7), or clathrin-mediated endocytosis (step 8) with recycling via endosomes (step 9). The steps in exocytosis are indicated by red arrows and the steps in endocytosis and recycling are indicated by yellow arrows (Sudhof, 2004).

The key event in the synaptic vesicle cycle is exocytosis by membrane fusion. For this to happen, several steps occur both before and after exocytosis. The synaptic vesicle cycle can be divided into 9 steps , as depicted in figure 1.2 (Sudhof, 1995; Sudhof, 2004)

1) NEUROTRANSMITTER UPTAKE: Neurotransmitters are taken up in synaptic vesicles driven by an electrochemical gradient created by a proton pump.

2) CLUSTERING AT THE ACTIVE ZONE: Synaptic vesicles cluster in front of the active zone.
3) DOCKING: Synaptic vesicles filled with neurotransmitters attach to the active zone of the presynaptic membrane.
4) PRIMING: After docking, synaptic vesicles go through a maturation process that makes them competent for fast Ca^{2+} triggered membrane fusion.
5) FUSION/EXOCYTOSIS: Primed synaptic vesicles are stimulated for rapid fusion/exocytosis by a Ca^{2+} spike during an action potential. Neurotransmitters are released in less than 1 msec.

After fusion-pore opening, synaptic vesicles endocytose and recycle probably by three alternative pathways, "kiss and stay", "kiss and run" or budding from an endosomal intermediate:

6) "KISS AND STAY": Vesicles are reacidified and refilled with neurotransmitters without undocking, thus remaining in the readily releasable pool.
7) "KISS AND RUN": Vesicles undock and recycle locally to reacidify and refill with neurotransmitter (back to step 1 and 2).
8) VESICLE ENDOCYTOSIS VIA CLATHRIN COATED PITS: Synaptic vesicles endocytose via clathrin coated pits and reacidify and refill with neurotransmitters directly.
9) RECYCLING VIA ENDOSOMES: Synaptic vesicles pass through an endosomal intermediate, and refill with neurotransmitters.

The synaptic vesicle cycle is unique in its speed and its tight regulation, due to the fact that one cycle takes approximately 60 sec to be finished (Siegel George J. et al., 1999).

Several proteins of synaptic vesicles have been identified, dividing them into transport proteins, membrane proteins, and proteins involved in neurotransmitter release. Transport proteins are proteins involved in the uptake of neurotransmitters. Trafficking proteins are both membrane proteins and proteins involved in neurotransmitter release. They mediate membrane traffic of the vesicles, such as docking, fusion and budding (Sudhof, 1995; Sudhof, 2004). The structure of trafficking proteins discovered so far is shown in figure 1.3.

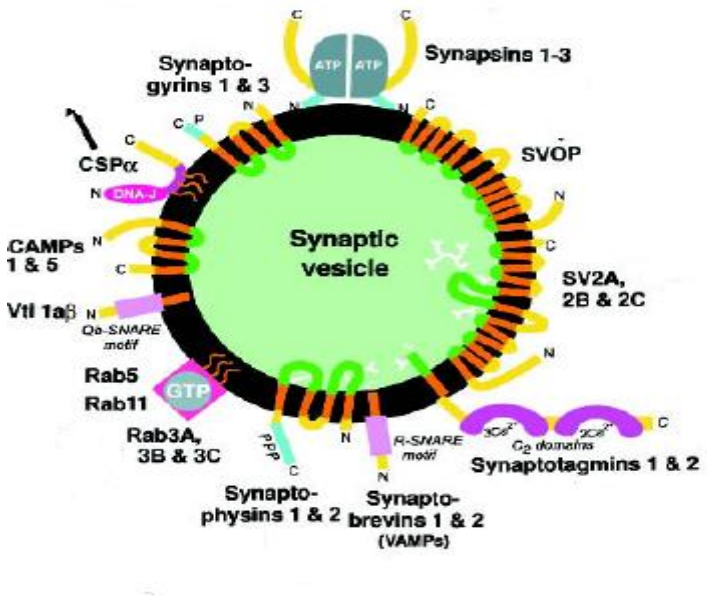


Figure 1.3 The structure of major trafficking proteins of synaptic vesicles that have been molecularly characterized (Sudhof, 2004)

Two trafficking proteins were examined in this investigation, namely synaptophysin and synapsins. Since the synapsins are described in detail in section 1.5, the main focus here is on synaptophysin. Synaptophysin is a membrane glycoprotein of synaptic vesicles that is ubiquitously expressed in all neurons and in many endocrine cells, accounting for 6–8% of the total synaptic vesicle proteins (Jahn *et al.*, 1985). It is currently the most widely used marker for nerve terminals. Synaptophysin 1 has four transmembrane domains with both N- and C-terminus facing the cytoplasm, as shown in figure 1 (Jahn *et al.*, 1985). Based on the predicated structure, it was suggested that synaptophysin forms a channel in the synaptic vesicle membrane and acts as the major Ca^{2+}-binding protein in synaptic vesicles (Gincel and Shoshan-Barmatz, 2002). Indeed, it has been demonstrated that upon reconstitution into a planar lipid bilayer, purified synaptophysin displayed voltage-sensitive channel activity (Gincel and Shoshan-Barmatz, 2002). The function of synaptophysin is, however, as yet unknown (Sudhof, 2004). On the one hand, its location in the synaptic vesicle membrane and its interaction with vesicle-associated membrane protein, VAMP (also known as synaptobrevin), implicated in synaptic vesicle docking and fusion, suggests its involvement in exocytosis (Gincel and Shoshan-Barmatz, 2002). However, the function of synaptophysin in neurotransmitter release has been questioned because mutant mice lacking synaptophysin displayed normal synaptic transmission (Gincel and Shoshan-Barmatz, 2002). As typical for synaptic vesicle proteins, synaptophysin represents a small protein family with three members, synaptophysin 1, synaptoporin (synaptophysin 2) and

panthophysin (Leube, 1994). Like synaptophysin 1, synaptoporin is widely expressed in neurons and colocalizes with synaptophysin 1 on synaptic vesicle whereas panthophysin is expressed in all tissues (Leube, 1994).

1.2 NEUROTRANSMITTERS

Neurotransmitters are released from synaptic vesicles when Ca^{2+} influx through voltage-gated channels. This release of neurotransmitters can be enhanced either by increasing the number of active synaptic vesicles, or by increasing the probability for the single docked vesicle to fuse (Sudhof and Jahn, 1991). The neurotransmitters of interest in this study are discussed in chapter 1.2.1 – 1.2.4.

1.2.1 γ - AMINOBUTYRIC ACID

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain and serves signalling and trophic functions in several neuronal tissues (Masson *et al.*, 1999). The rate-limiting step in the synthesis of GABA is the decarboxylation of glutamate, a reaction catalyzed by the enzyme glutamic acid decarboxylase (GAD) (Bu *et al.*, 1992). GABA taken up into nerve terminals can be reutilized, while GABA taken up in glia is metabolized to succinic semialdehyde by GABA transaminase. and cannot be resynthesized to GABA since glia cells lack GAD (Siegel George J. et al., 1999).

High levels of GABA are found within the neocortex, hippocampus, cerebellum, septal nuclei and the reticular nucleus of the thalamus (McIntire *et al.*, 1997).

A growing body of evidence suggests a role for altered GABAergic function in neurological and psychiatric disorders of humans, including Huntington's desease, epilepsy, tardive dyskinesia, alcoholism, schizophrenia, sleep disorders, Parkinson's disease and mental retardation (Siegel George J. et al., 1999). This is the reason why GABAergic transmission is an important therapeutic target.

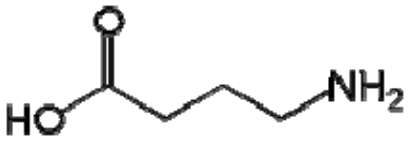


Figure 1.4 The molecular structure of GABA

Since GAD65 was examined in this investigation, a brief description of GAD isoforms will be presented here. GAD exists as two major isoforms, called GAD65 and GAD67, which are the products of two independently regulated genes located on chromosomes 2 and 10, respectively, in humans (Bu *et al.*, 1992). The N-terminal domain (with 23% identity between the two human GADs, as depicted in figure 1.5) is involved in subcellular targeting, membrane association and heteromeric interactions. The C-terminal is much larger and more conserved (73% identity, as depicted in figure 1.5), having a domain that contains the catalytic centre (Soghomonian and Martin, 1998). Membrane association of GAD65 involves phosphorylation of the N-terminal domain and other interactions (Soghomonian and Martin, 1998). Membrane association of GAD67 appears to be secondary to that of GAD65 and involves the formation of GAD65–GAD67 heteromers through undefined interactions between the N-terminal domains of the two forms (Kanaani *et al.*, 1999). These differences in membrane interactions might account for the differences in subcellular localization of the two GAD forms in the brain, and they could be involved in the biosynthesis of GABA in different intracellular compartmnts (Soghomonian and Martin, 1998). Although both isoforms synthesize the neurotransmitter GABA, GAD67 might preferentially synthesize cytoplasmic GABA and
GAD65 might preferentially synthesize GABA for vesicular release.

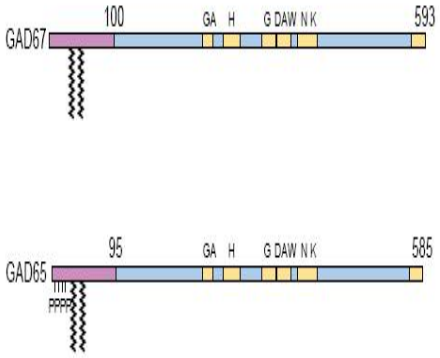


Figure 1.5 Comparison of glutamate decarboxylase 65 (GAD65) and GAD67 proteins. The N-terminal
domain is on the left side.. Phosphorylation of four serines near the N-terminus of GAD65 appears to be involved in membrane association. The catalytic domain of the two GAD proteins is more highly conserved (73% sequence identity). It contains six motifs that are structurally conserved in the GADs and related pyridoxal-phosphate-dependent enzymes and also several conserved residues that interact directly with the cofactor (Soghomonian and Martin, 1998)**.**

1.2.2 GLUTAMATE

The amino acid glutamic acid, also referred to as glutamate, is the major excitatory neurotransmitter, found in large amounts in the central nervous system (CNS). It is synthesised in neuronal tissues either by transamination of α-ketoglutarate or from glutamine by the action of glutaminase. Glutamate mediates through the interaction with a large, and still growing number of receptors, both ionotropic and metabotropic, fast excitatory neurotransmission as well as synaptic plasticity. Since glutamate does not cross the blood-brain barrier, it is stored within nerve terminals in virtually every neuron in the CNS. When taken up into glial cells, glutamate is converted into glutamine (Siegel George J. et al., 1999).

Elevated levels of glutamate can induce severe damages to target neurons due to its characteristic as being a potent exitotoxin. It's removal from the synaptic cleft is of key importance to maintain the integrity of neuronal tissue (Masson *et al.*, 1999). Since elevated levels of glutamate has been implicated in epilepsy, ischemic brain damage and learning (Siegel George J. et al., 1999), glutamatergic pathways are of great therapeutic interest.

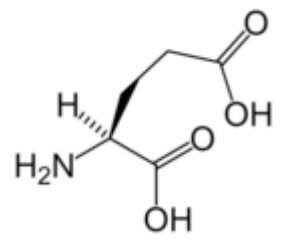


Figure 1.6 The molecular structure of glutamate

1.2.3 ACETYLCHOLINE

Acetylcholine is one of the major modulator of brain functions. The synthesis of acetylcholine is a single step reaction catalyzed by the enzyme choline acetyltransferase (ChAT) using acetyl coenzyme A and choline taken up by the high affinity Na^+-dependent choline transporter (Prado *et al.*, 2002). Acetylcholine is metabilised by cholinesterases, acetylcholinesterase (AChE) or butyrylcholinesterase (Siegel George J. et al., 1999)

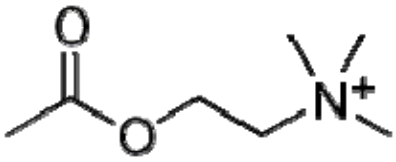


Figure 1.7 The molecular structure of acetylcholine

As shown in figure 1.8 of central cholinergic pathways in rat brain, forebrain projections are classified into six main central pathways (Ch1–Ch6), linked to the origin of the nuclei where the cholinergic fibres arise. Cholinergic nuclei from the septum (Ch1) and the vertical limb of the diagonal band (Ch2) project only on the hippocampus whereas pedunculopontinus nucleus (part of Ch5) and laterodorsal tegmental nucleus (Ch6) from the brainstem project on the thalamus. Cholinergic nuclei from the lateral part of the horizontal limb of the diagonal band (Ch3) project to the olfactory bulb. The pathway innervating the cortex (Ch4) mainly originates from the nucleus basalis magnocellular. The only difference between cholinergic projecetions in human brain compared to rat brain is that in the rat, Ch4 pathway groups originate from several other nuclei in addition to the nucleus basalis magnocellular, such as the substantia innominata, diagonal band nuclnucleus ansa lenticularis and a part of the magnocellular preoptic nucleus (Lucas-Meunier *et al.*, 2003). In contrast, the neostriatal regions in the basal ganglia (caudatoputamen, nucleus accumbens and olfactory tubercle) contain local cholinergic interneurons only.

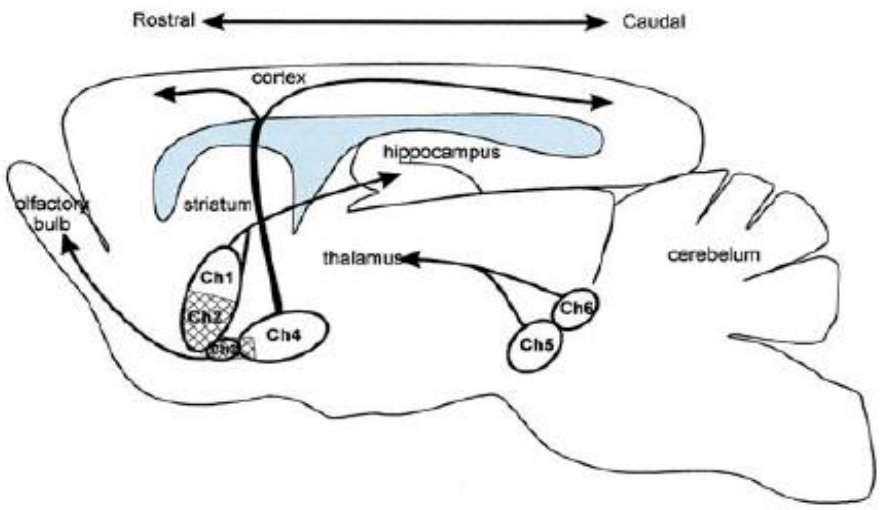


Figure 1.8 Rat central cholinergic pathways (Lucas-Meunier *et al.*, 2003).

Acetylcholine acts as a neurotransmitter in the peripheral nervous system (PNS) as well. Muscarinic receptors are found in visceral smooth muscle, in cardiac muscle, in secretory

glands and in the endothelial cells of the vasculature. Except for endothelial cells, each of these sites receives cholinergic innervation. The responses can be excitatory or inhibitory, depending on the tissue (Siegel George J. et al., 1999).

Modulation of acetylcholine release is crucial for the function of the nervous system. Dysfunction of cholinergic transmission has been linked to a number of pathological conditions (Prado *et al.*, 2002), such as Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob disease, Down syndrome, Korsakoff's syndrome. (Lucas-Meunier *et al.*, 2003).

1.2.4 DOPAMINE

Dopamine is the major catecholamine in the CNS (Jaber *et al.*, 1996), and it has been shown that the dopaminergic system mediates control of movements, hormone secretion, cognitive, emotional and reward behaviours (Brusa, 1999).

Dopamine is synthesized by the hydration of the amino acid tyrosine to DOPA by tyrosine hydroxylase, being the rate-limiting step. Thereafter DOPA is decarboxylated by aromatic-L-amino-acid decarboxylase. The catechol-O-Methyltransferase (COMT) and monoamine oxidase (MAO), catalyses degradation of catecholamines (Siegel George J. et al., 1999).

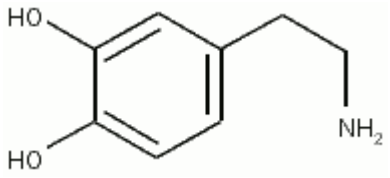


Figure 1.9 The molecular structure of dopamine

The dopaminergic system consists of three major pathways. The nigro-striatal branch originate in the substantia nigra and project mainly to the neostriatum (the caudate-putamen complex). Nearly 80% of all dopamine is found in neostriatum. The meso-cortico and the meso-limbic branch originate in the ventral tegmental area and project to the prefrontal cortex, the nucleus accumbens septi and the olfactory tubercle (Siegel George J. et al., 1999).

The dopaminergic system has been thoroughly studied, mainly because alterations in dopamine neurotransmission are involved, directly or indirectly, in several brain

dysfunctions, e.g. schizophrenia, Parkinson's disease, Attention-deficit hyperactivity disorder (ADHD), Huntington's disease, Tourettes syndrome and in drug addiction (Castellanos and Tannock, 2002; Jaber et al., 1996).

1.3 NEUROTRANSMITTER TRANSPORTERS

Neurotransmitter transporters can be classified according to their primary structure and site of action. According to this criterion two families of neurotransmitter transporters have been classified, namely plasma membrane transporters and vesicular membrane transporters (Masson *et al.*, 1999). Vesicular neurotransmitter transporters are described in greater detail then plasma membrane neurotransmitter transporters, since vesicular transporters are of great interest in this study.

1.3.1 **PLASMA MEMBRANE NEUROTRANSMITTER TRANSPORTERS**

Plasmamembrane transporters are responsible for neurotransmitter uptake at the plasma membrane, where all transporters are dependent on the Na^+ gradient for their activity. Depending on whether they need Cl^- or K^+ in addition to be active, these transporters can further be classified as Na^+/Cl^--dependent or Na^+/K^+-dependent neurotransmitter transporters. The monoamine (dopamine, norepinephrine and serotonin) and inhibitory amino acid (e.g. GABA) transporters are all Na^+/Cl^--dependent transporters, while excitatory glutamate and aspartate are Na^+/K^+-dependent transporters. There are differences in these two classes of transporters, amongst others in their pharmacological properties, regulatory properties, regional and cellular localizations and implications in neuropathologies (Masson *et al.*, 1999).

1.3.2 **VESICULAR NEUROTRANSMITTER TRANSPORTERS**

At present, vesicular transporters for the monoamines (VMAT-1 and VMAT-2), acetylcholine (VAChT), GABA (VGAT) and glutamate (VGLUT-1, VGLUT-2 and VGLUT-3) have been described (Fykse and Fonnum 1988; Fykse and Fonnum 1996; Maycox *et al*., 1990). Synaptic vesicles accumulate and store neurotransmitters at high concentrations by active transport, driven by a vacuolar proton (H^+) pump. This ATP-driven H^+-pump, energize neurotransmitter uptake by proton pumping which acidifies the organelle lumen, hereby generating a pH difference (ΔpH), or in the absence of proton generates a large membrane potential ($\Delta\Psi$) (Maycox, Hell, and Jahn, 1990). Synaptic vesicles express specific and distinct neurotransmitter transporters which define transmitter

specificity and amount of transmitter in the vesicles (Sudhof and Jahn, 1991).These proteins are all ATP-dependent, and sequence analyses have predicted a structure with 10-12 membrane-spanning domains. However, the driving force behind the vesicular transport of amino acid transmitters differs from the transport of monoamines and ACh. While VMATs and VAChT chiefly rely on the pH gradient (ΔpH) across the vesicle membrane to drive active transport of neurotransmitter into the vesicle, vesicular glutamate transport depends primarily on the electrical gradient and vesicular GABA transport depends more equally on both ΔpH and $\Delta\psi$.

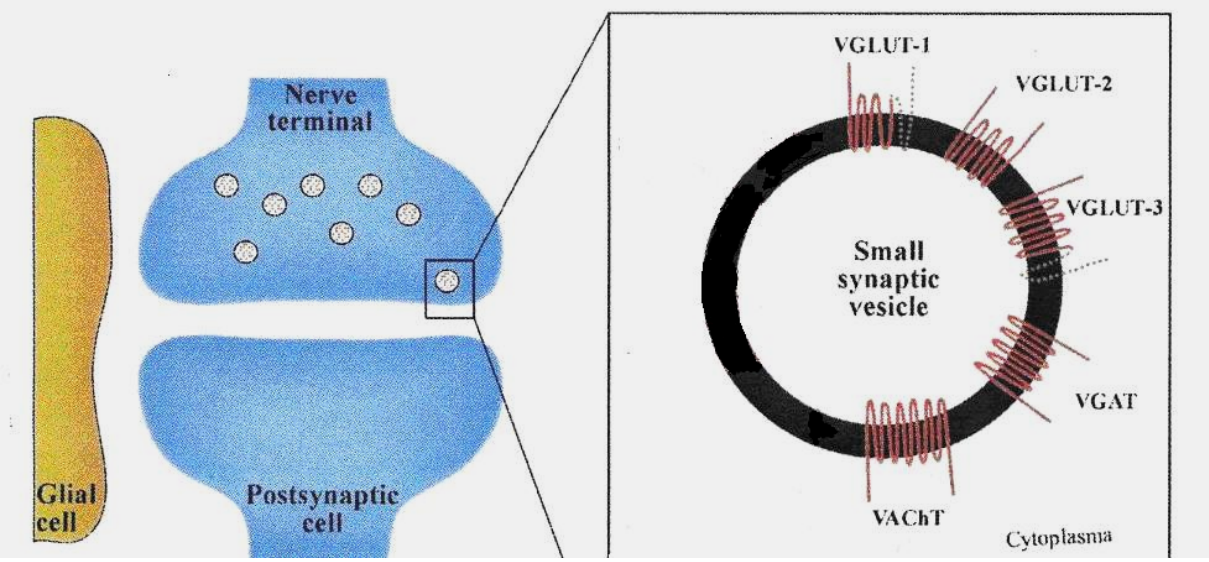


Figure 1.10: The structure of vesicular neurotransmitter transporters of synaptic vesicles are depicted schematically. For simplicity, different vesicular transporters are pictured in the same synaptic vesicle although they do not colocalize in situ. Based on (Gasnier, 2000; Masson et al., 1999; Maycox, Hell, and Jahn, 1990).

VESICULAR GABA TRANSPORTER

The vesicular GABA transporter (VGAT) is a protein with ten transmembrane domains, expressed in regions containing GABAergic neurons. It has a long NH_2- and a short COOH-intracytoplasmic termini, as shown in figure 1.10. (McIntire *et al.*, 1997; Sagne *et al.*, 1997).

VGAT is responsible for the uptake and storage of GABA by synaptic vesicles in the central nervous system, although it appears to be responsible also for the uptake of glycine (McIntire *et al.*, 1997; Sagne *et al.*, 1997). VGAT is different from the plasma membrane transporters in that it is driven by a proton electrochemical gradient across the vesicle

membrane. The substrate affinity for this transporter is low (Gasnier, 2000; Maycox, Hell, and Jahn, 1990). So far, only one isoform of VGAT is known.

VESICULAR GLUTAMATE TRANSPORTER

There are three known vesicular glutamate transporters (VGLUTs), namely VGLUT-1, VGLUT-2 and VGLUT-3 (Fremeau *et al.*, 2004). VGLUT-1 have six transmembrane domains, while VGLUT2 and VGLUT3 have eight transmembrane domains, all with N- and C-terminal regions facing the cytosol.VGLUT-1 and VGLUT-2 are both necessary and sufficient for uptake and storage of glutamate and thus comprises the sole determinant for a glutamatergic phenotype. Both VGLUTs are different from the plasma membrane transporters in that they are driven by a proton electrochemical gradient across the vesicle membrane. VGLUT1 and VGLUT2 show complementary expression patterns. In contrast VGLUT 3 defines a new distinct glutamatergic system in brain which is strictly seperated from VGLUT 1 and VGLUT 2 synapses. Co-localization with the acetylcholine transporter VAChT and the monoamine transporter 2 VMaT 2 has been observed (Fremeau *et al.*, 2002; Gras *et al.*, 2002).

VESICULAR ACETYLCHOLINE TRANSPORTER

Vesicular acetylcholine transporter (VAChT) is a 12 transmembrane transporter, with N- and C-terminal regions directed to the cytosol, as depicted in figure 1.10 (Roghani *et al.*, 1994). VAChT is a selective marker of cholinergic neurons, localized in small, clear synaptic vesicles of axon terminals (Prado *et al.*, 2002). This transporter catalyzes the exchange of 2 H^+ per cationic transmitter, and chiefly relies on proton gradient (Gasnier, 2000; Prado *et al.*, 2002).

Both VAChT and ChAT are encoded by two embedded genes, the VAChT gene lying within the first intron of the ChAT gene, being co-expressed. This unique organization was named “cholinergic gene locus”, and it has been shown that ChAT and VAChT proteins are co-expressed (Prado *et al.*, 2002)

In the PNS, VAChT was detected in motor endplates of skeletal muscles as well as in fibers of sympathetic and parasympathetic abdominal ganglia, heart atrium, respiratory tract, and salivary and lacrimal glands (Arvidsson *et al.*, 1997), indicating that VAChT can

be used as a tool for the study of cholinergic neurons in the central and peripheral nervous systems.

1.4 NEUROTRANSMITTER RECEPTORS

Dopamine D_1-like receptors and NMDA-receptors have been examined in this study and are therefore described in more detail.

1.4.1 DOPAMINE RECEPTORS

Five dopamine receptors have been cloned and pharmacologically classified in two receptor subfamilies; D_1 and D_2. The D_1 subfamily comprises the D_1 (or D_{1A}) and D_5 (or D_{1B}) receptors, and are classified as D_1-like receptors. The D_2 subfamily includes the D_2, D_3, and D_4 receptors, and are classified as D_2-like receptors (Brusa, 1999; Jaber *et al.*, 1996). All five receptors are members of the large G-protein coupled receptor superfamily, consisting of seven transmembrane domains. The members of the same family share common characterics, such as their general folding pattern and structure, which govern receptor interactions with ligands, as well as with proteins of signal transduction pathways. The main difference between these receptor subfamilies, is that D_1 like receptors stimulate adenylyl cyclase, while D_2-like receptors inhibit the activity of adenylyl cyclase, as depicted in figure 1.11 (Jaber *et al.*, 1996).

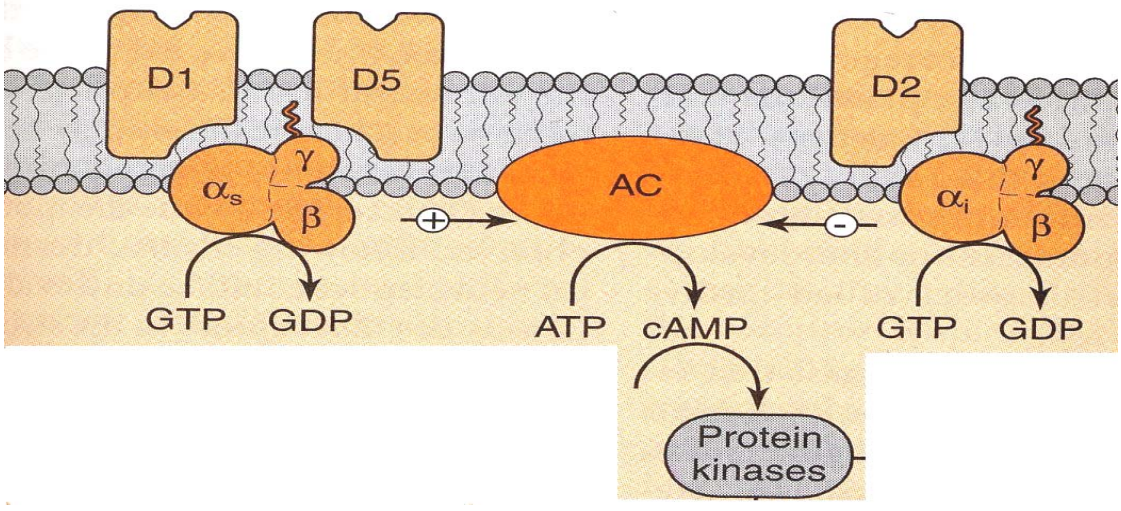


Figure 1.11: Agonist binding to D_1-like receptors (D_1 and D_5) activates trimeric G-protein (α_s, β, γ). This activates adenylyl cyclase (AC), which in turn catalyzes the conversion of ATP to cAMP, activating protein kinase. Binding of D_2-like receptors (D_2, D_3 and D_5) activates an inhibitory G-protein (α_i, β, γ), thereby inhibiting adenylyl cyclase (Siegel G.J. *et al.*, 1999).

As mentioned above D_1-like receptors couple to G_s, a stimulatory G-protein, stimulating adenylyl cyclase, and have the classical D_1 pharmacology. Activated adenylyl cyclase catalyzes the conversion of ATP to cyclic AMP (cAMP), which in turn causes dissociation of the regulatory and catalytic subunit of protein kinase A (PKA). This enzyme catalyzes conversion of protein substrates to phosphoproteins (Siegel G.J. *et al.*, 1999). Although they share very high homology within their transmembrane domains, the D_5 receptor has 10-fold higher affinity for dopamine compared to D_1(Jaber *et al.*, 1996). The D_1 receptor is the most widespread dopamine receptor and is expressed at a higher level than any other dopamine receptor, being found in the striatum, nucleus accumbens and olfactory tubercle. The D_1 receptor is also found in the limbic system, hypothalamus and thalamus. The D_5 receptor is expressed at a much lower level than the D_1 receptor, being restricted to the hippocampus (Jaber *et al.*, 1996). Many D_1 receptor interacting proteins have been described, amongst others calcyon, a single pass transmembrane protein thought to play an important role in D_1 receptor Ca^{2+} signalling (Lezcano *et al.*, 2000).

The D_2, D_3 and D_4 receptors are considered to be D_2-like because of their homology and pharmacology. They are coupled to an inhibitory G-protein (G_i), and inhibit adenylyl cyclase. The D_2 receptor is the dominating receptor of the D_2-like receptors, and is found mainly in the neostriatum, olfactory tubercle and nucleus accumbens. The D_3 and D_4 receptors are restricted to limbic areas (Jaber *et al.*, 1996).

1.4.2 *N*-METHYL-D-ASPARTATE RECEPTOR

The N-methyl-D-aspartate (NMDA) receptor is an ionotropic, glutamate receptor which contains four transmembrane segments following a large extracellular domain (Moriyoshi *et al.*, 1991). This receptor has binding sites for both glutamate and modulatory glycine (Moriyoshi *et al.*, 1991).

There are six known NMDA- receptor subunit genes, encoding NMDA-1, NMDA-2A-NMDA-2D and NMDA-3A subunits.The NMDA-1 subunit is regarded as an obligatory subunit since it is required for the formation of functional NMDA- receptor channels, while the NMDA-2 subunit is a modulatory subunit. The NMDA-3 subunit is expressed only in early development (Popescu, 2005; Stephenson, 2001). While glycine binds to the NMDA-1 subunit, glutamate is bound to the NMDA-2 subunit (Popescu, 2005; Stephenson, 2001).

Functional NMDA-receptors are formed by the co-expression of NMDA-1 and NMDA-2 subunits as shown in figure 1.12, yielding receptors with different biophysical and pharmacological properties (Popescu, 2005; Stephenson, 2001).

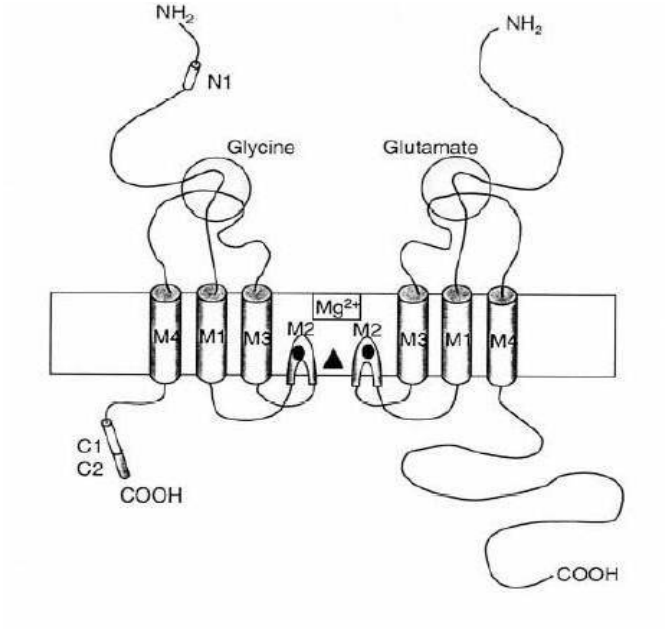


Figure 1.12 : A schematic diagram showing predicted transmembrane topology of an NMDA-1 (left) and NMDA-2 subunit where the N-terminal is extracellular (Stephenson, 2001)

NMDA receptors are expressed throughout the whole brain (Popescu, 2005; Stephenson, 2001). High levels of NMDA receptors are implicated in seizures and neuronal loss, while low levels are implicated in schizophrenia, indicating it's involvement in neurological disorders (Moriyoshi *et al.*, 1991; Stephenson, 2001).

1.5 THE SYNAPSINS

Synaptic vesicles are coated by synapsins, phosphoproteins that account for 9% of the vesicle protein (Kao *et al.*, 1999; Rosahl *et al.*, 1995). As described in chapter 1.1.2, these proteins are involved in the synaptic vesicle cycle (Sudhof, 2004). The synapsins are a family of neuron-specific proteins that are concentrated at synapses, where they are bound to the cytoplasmic surface of synaptic vesicles, by their N-terminal, as depicted in figure 1.3 (Hosaka, Hammer, and Sudhof, 1999; Kao et al., 1999; Sudhof, 2004).

In mammals, three synapsin genes, I, II, and III, have been cloned and characterized in human, mouse, and rat (Kao *et al.*, 1998; Sudhof, 2004). The precise chromosomal locations of the genes for human and mouse synapsins I (Yang-Feng, DeGennaro, and Francke, 1986), II (Li *et al.*, 1995a; Li *et al.*, 1995c) and III (Kao *et al.*, 1998) have now

been mapped. Human and mouse synapsin I map to the X chromosome, synapsin II maps to 3p25 and 6F, and synapsin III maps to 22q12.1 and 10, respectively.

1.5.1 PRIMARY STRUCTURE AND PHYSIOCHEMICAL PROPERTIES OF THE SYNAPSINS

Alternative splicing of the primary transcripts encoded by the three synapsin genes yields the five known synapsins: Ia, Ib, IIa, IIb and IIIa, as shown in figure 1.13. The differences between the 'a' and 'b' isoforms seem to be restricted to the C-terminal region (Ferreira and Rapoport, 2002).

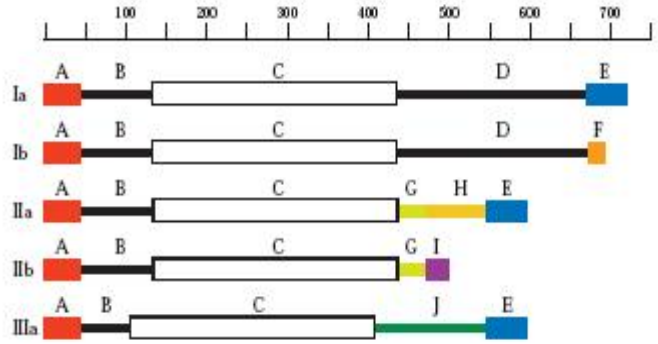


Figure 1.13 Mammalian domain model of the synapsins: Domains are schematically represented and indicated by letters A–J. The length of the polypeptide chains is shown at the top in number of amino acid residues (Kao *et al.*, 1999).

The common regions for all synapsin proteins are domains A to C, of nine known domains, as shown in figure 1.13 (domain A-J) (Kao et al., 1999; Valtorta, Benfenati, and Greengard, 1992). The domains E, F, H and I represent the portions of the molecules generated by alternative splicing (Valtorta, Benfenati, and Greengard, 1992), although domain E is a common component of both the synapsin Ia, synapsin IIa and synapsin IIIa isoforms (Ferreira and Rapoport, 2002).

The different protein domains confer both to different function as well as different structural elements for synapsin proteins. In brief, domain A is a phosphorylation site for both cAMP-dependent protein kinase and Ca^{2+}/calmodulin dependent protein kinase I (CaM kinase I) (Hilfiker et al., 1999; Valtorta, Benfenati, and Greengard, 1992). Domain C exhibits the greatest similarity between synapsin proteins. The C domain binds ATP in all synapsin proteins and binds to other C-domains, mediating the formation of both homo- and heterodimers of synapsins(Ferreira and Rapoport, 2002; Valtorta, Benfenati, and

Greengard, 1992). Domain D is present only in synapsin I and contains two phosphorylation sites for CaM kinase II (Valtorta, Benfenati, and Greengard, 1992).

1.5.2. THE FUNCTION OF THE SYNAPSINS

Although the synapsins were among the first vesicle proteins to be discovered (Sudhof, 2004), their functions still remain incompletely understood.

The synapsin protein family is believed to play important roles in the generation and maintenance of synaptic vesicle clusters (Hilfiker *et al.*, 1999). Many studies have shown that synapsins mediate the attachment of synaptic vesicles to actin filaments, and thereby regulate the amount of synaptic vesicles in the releasable pool. The interactions between synapsins and the cytoskeleton are reversible, due to phosphorylation of synapsins which decreases anchoring of synaptic vesicles to the cytoskeleton. Phosphorylation and dephosphorylation states of synapsins are suggested to affect the amount of synaptic vesicles being readily releasable or stored in reserve pool (Hilfiker et al., 1999; Valtorta, Benfenati, and Greengard, 1992). Synapsins may also modulate neuronal development, e.g. neurite elongation, establishment of neuronal polarity and synapse formation and maintenance, where each synapsin protein is involved at different stages of development (Bogen et al., 2006; Ferreira and Rapoport, 2002).

1.6 ANIMAL MODELS

In this study, two different animal models were used, namely synapsin I/II DKO mice, and an animal model for ADHD, Spontaneously Hypertensive Rat (SHR). Due to this reason gene knockout technology behind synapsin I/II DKO mice will be described as well as the breeding process behind SHR rats.

1.6.1 GENE KNOCKOUT TECHNOLOGY

Genetically engineered mice have become an invaluable biological tool for better understanding of physiological as well and pathological processes in biomedical research. In the field of neurosciences these animals have given a great contribution to shed light on basic mechanisms of brain function, e.g. elucidating physiological function of specific proteins such as receptors or intracellular mediators. In principle there are two ways to generate mutant mice, either by pronuclear microinjection or by homologous recombination in embryonic stem (ES) cells (Brusa, 1999). Since homologous

recombination was used for generation of the synapsin I/II DKO mice, this technique will be discussed further.

The most widely used gene-targeting strategy is the production of complete loss-of-function mutations (null mutations), or so called gene knockout (KO). In brief, a targeting vector carrying a selectable marker flanked by a sequence homologous to the genomic target gene is constructed and introduced by transfection into an ES cell line. Cells in which homologous recombination has occurred are injected into host blastocysts, which are reimplanted in the uterus of peseudopregnant foster mothers (Brusa, 1999; Galli-Taliadoros *et al.*, 1995; van der Neut, 1997). If ES cells contributed to the germ line the mutation is transmitted to the offspring and by subsequent matings homozygous mice are generated (Brusa, 1999).

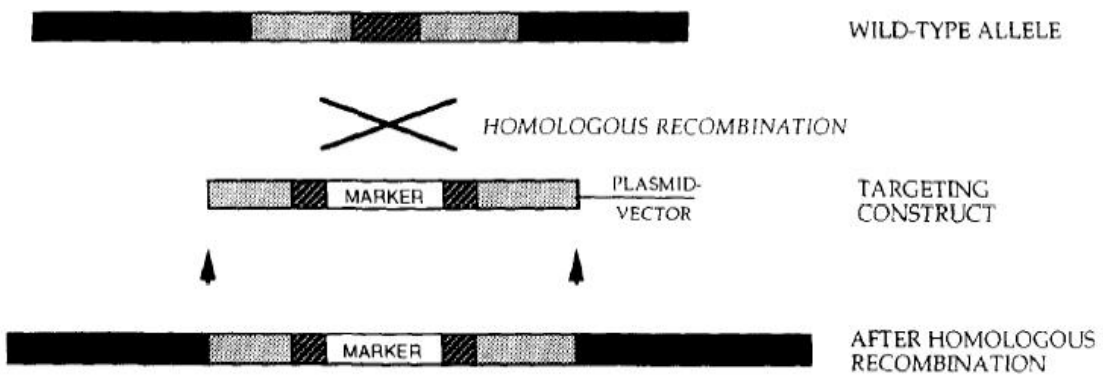


Figure 1. Homologous recombination: The underlying concept of homologous recombination as described by Galli-Taliadoros *et al. (*1995)

1.6.2 THE SPONTANEOUSLY HYPERTENSIVE RAT

The spontaneously hypertensive rat (SHR) was developed in Kyoto, Japan, from the progenitor Wistar-Kyoto (WKY) rat by inbreeding [1]. SHR is a commonly used animal model in studies of hypertension. During the inbreeding of the SHR for the high blood pressure trait, several behavioural characteristics were also seen, among others behavioural hyperactivity, increased beheavioral response to stressful stimuli, slower habitutation to novel stimuli and faster acquisition of active avoidance (Sagvolden, Hendley, and Knardahl, 1992).

[1] Inbreeding is breeding between close relatives, whether plant or animal.

It has repeatedly been suggested that SHR might be used as an animal model of Attention deficit disorder (ADHD) (Sagvolden, 2000; Sagvolden et al., 1992), a disorder prevalent during childhood, characterized by impared attention, excessive motor activity and impulsivity (Adriani *et al.*, 2003). SHR develops response bursts similar to ADHD children, and although other strains and species may be hyperactive and/or show attention deficits following genetic, environmental or pharmacological interventions, the SHR is presently the only strain shown to have the major behavioral symptoms of ADHD (Sagvolden, 2000; Sagvolden and Sergeant, 1998). Since there are many questions left regarding the ADHD disorder, SHR is of great importance in ADHD research.

1.7 AIMS OF THE STUDY

This study consists of two different parts. In the first part we have pursued the study of Bogen *et al*., (2006) with the main focus on what happens to levels of VAChT in mice devoid of synapsin I and II. Based on these findings, it was of interest to study if synapsin also serve a role in the cholinergic nervous system and therefore to see whether synapsin I/II DKO mice have changed levels of VAChT in the CNS and PNS, compared to their control wild-type (WT) mice. Three cholinergic brain areas/loci were selected, namely the neostriatum, cortex and pons medulla. The levels of other vesicular subgroups of synaptic vesicles, such as the glutamatergic and GABAergic vesicles, were also examined in these brain areas of synapsin I/II DKO mice.

The aims of the second parts of the study were to determine the levels and possibly activities of proteins functionally related to specific neurotransmission systems in animal models for the "attention deficity hyperactivity syndrome" ADHD, SHR Due to that dopaminergic system has been hypothesized to be involved in the development of ADHD (Davids *et al.*, 2003; Oades *et al.*, 2005; Russell, 2003; Sagvolden *et al.*, 2005), it was of interest to see whether the level of dopamine D_1-receptor in SHR was changed in the neostriatum and cerebrum, and whether dopamine interacting proteins were changed as well.

2. MATERIALS AND METHODS

2.1 MATERIALS

Summary of chemicals and reagents, as well as content of buffers and solutions used in this assay are presented in the APPENDIX.

2.2 ANIMALS

Two different animal models were used in this study as indicated in 1.6.

2.2.1 SYNAPSIN I/II DOUBLE KNOCKOUT MICE

Mice devoid of synapsin I and II were obtained by homologous recombination as described in 1.5.1. The animals were a gift from Dr Paul Greengard (The Rockefeller University, NY, USA). The experimental animals were kept under conditions of constant temperature (22± 2^{o}C) and humidity (55 ± 5%), a 12 h light/dark cycle and free access to food and water.

The animals were treated according to the Norwegian Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2.2 SPONTANEOUS HYPERTENSIVE RATS

25 Male Wistar Kyoto (WKY) and 25 Male Spontaneous Hypertensive Rats (SHR) were purchased from Harlan, England, and Charles River, Germany, respectively. 15 WKY and 15 SHR were 4 weeks old, while 10 WKY and 10 SHR were 8 weeks old. The animals were kept under conditions of constant temperature (22± 2^{o}C) and humidity (55 ± 5%), a 12 h light/dark cycle and free access to food and water.

The animals were treated according to the Norwegian Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3 SAMPLE PREPARATION

Due to different laboratory methods various procedures for three sample preparations were carried out. These are as following:

2.3.1 PREPARATION OF CRUDE HOMOGENATE FROM RATS DEVOID OF SYNAPSIN I AND II

The neostriatum, pons/medulla oblongata and cerebral cortex, as well as the whole cerebrum, were dissected on ice and rapidly homogenized in 0.32M sucrose [5% homogenate (w/v)] in a glass-Teflon homogenizer at 450 rpm[2]. Samples were then added sodium dodecyl sulphate (SDS) (final concentration 1 %, w/v), boiled at 100^{o}C for 3 minutes, and stored at - 40^{o}C.

2.3.2 PREPARATION OF CRUDE HOMOGENATE FROM WKY AND SHR RATS

Striatum and cerebrum were dissected on ice. They were rapidly homogenized in 0.32M sucrose [5% homogenate (w/v)] in a glass-Teflon homogenizer at 450 rpm. Samples were then added to SDS (final concentration 1 %, w/v), and stored at - 40^{o}C.

2.3.3 PREPARATIONS OF HOMOGENATE FROM DIAPHRAGM AND TONGUE

Due to pilot studies that were carried out on vAChT and the synaptic vesicle protein synaptophysin in the peripheral nervous system of mice devoid of synapsin I and II proteins, different procedures for homogenate preparations of diaphragm and tongue were made, making it suitable for Western blotting.

PROCEDURE 1

Mice were killed by cervical dislocation. After dissection, both tongue and diaphragm were frozen on liquid nitrogen. Diaphragm and tongue muscles were cut with a razorblade, and homogenized in a glass-glass homogenizer at 450 rpm with homogenization buffer (0.32 M sucrose with 10mM HEPES and 1 mM EGTA). The homogenate (10% w/v) was centrifuged at 3000 rpm for 10 minutes, and the supernatant collected. To 200 µl sample, SDS was added (final concentration 1 %), sample was and stored at -40^{o}C.

[2] rpm, or revolutions per minute, is a unit commonly used to measure rotational speed in standard centrifuges

PROCEDURE 2

Mice were killed by cervical dislocation. After dissection, both tongue and diaphragm were frozen on liquid nitrogen. Diaphragm and tongue muscles were cut with a razorblade, and homogenized in a glass-glass homogenizer at 450 rpm, with 0.03 % or 0.3 % Triton X-100 added to WT and KO samples from both diaphragm and tongue (final concentration 10 %). Homogenate aliquots[3] were centrifuged at 12 000 rpm for 20 minutes and supernatant collected. Supernatant was then added to SDS (final concentration 1 %), and stored at - 40^{o}C.

PROCEDURE 3

Mice were killed by cervical dislocation. After dissection, both tongue and diaphragm were frozen on liquid nitrogen. Diaphragm and tongue muscles were crushed in a mortar, and homogenized in 10mM HEPES in a glass-glass homogenizer rotating at 450 rpm, where three different diaphragms were combined, as well as three tongues from both WT and KO,. Homogenate was centrifuged at 9000 rpm for 10 minutes, the supernatant was collected and centrifuged at 400 000 rpm for 60 minutes. The pellet was re-suspended in dH_20 with SDS (final concentration 1 %). The supernatant was also added to SDS (final concentration 1 %). Both pellet and supernatant were boiled at 100^{o}C for 3-4 minutes, and stored at - 40^{o}C.

PROCEDURE 4

Mice were killed by cervical dislocation After the dissection, both tongue and diaphragm were frozen on liquid nitrogen. Diaphragm and tongue muscles were cut with a razorblade, and homogenized in a glass-glass homogenizer at 450 rpm with TCA/acetone (final concentration 1.5 % for diaphragm samples, and 1 % tongue for tongue samples). In each tube, 500 µl 1M KCL was added, and left to stand for 10 minutes at 4^{o}C. Homogenate was then centrifuged at 14 000 rpm for 5 minutes, supernatant flicked off and pellet of tongue and diaphragm sample re-suspended (into 0.5 ml and 1ml respectively of acetone/DTT/NaF (final concentration 10mM DTT, 20 mM NaF). Tubes were centrifuged again at 14 000 rpm, supernatant flicked off, and pellets vacuum dried for 20 minutes. Pellet of tongue and diaphragm sample were re-suspended in respectively 200 µl and 400 µl of urea sample buffer (final concentration 20 mM Tris, 22 mM Glycine, 8M Urea and 10 mM DTT, (pH

[3] One sample of KO of tongue and one sample of KO diaphragm were not centrifuged.

8.6). Samples were sonicated on ice 4 x 30 seconds, with 30 seconds between each sonication., left on for 2 h on rotor in 4^{o}C, vortexing sample after 30 minutes and 1 hour and 15 minutes. Sonication step was repeated, and samples were sonicated 2 x 30 seconds, with 30 seconds between each sonication. Sample was sheared by passing it through ice cold needle, and afterwards centrifuged at 14 000 rpm for 2-3 minutes. Supernatant was removed to clean eppendorf tubes, and samples were frozen at -40^{o}C.

PROCEDURE 5

Same procedure as in 2.2.3.3, except that diaphragm and tongue were homogenized in an UltraTurrax cutter _and protease inhibitor was added prior to homogenization, together with 10mM HEPES.

2.4 PROTEIN DETERMINATION

Protein concentration was determined as described in BCA ^{TM} Protein Assay Kit, Pierce, Rockford, IL, USA. In brief, BCA^{TM} Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. Proteins reduce Cu^{2+} in alkaline solutions to Cu^{+}. Two molecules BCA react with each Cu^{+} resulting in a red water soluble complex with an absorption maximum of 562 nm. Absorbance is proportional to the total protein concentration, which allows spectrophotometric quantification of protein in aqueous solutions.

In every protein determination, we used a microplate procedure, with seven standards of bovine serum albumin (BSA) (0, 0.125 mg/ml, 0.25mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1.0 mg/ml, 1.5 mg/ml) and four replicates of every sample. 200 µl of BCA–reagent was added in each well.

2.5 WESTERN BLOTTING

Western blotting is a method used for protein detection in a sample, after separation on a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). The basic blotting procedure can be divided into sample preparation, gel electrophoresis, membrane transfer, blocking non-specific binding, addition of the antibody and detection (Towbin *et.al.*, 1979).

Samples are loaded into the wells on a gel, which in turn is placed in an electrode assembly. SDS-PAGE gel electrophoresis will then separate proteins in complex mixtures according to size. Using electrophoresis, proteins are then transferred on to a nitrocellulose filter. Primary antibody will then attach to an epitope of the protein of interest. Secondary antibody will then bind to the primary antibody. This antibody, which binds to the primary antibody, is coupled to an enzyme, horseradish peroxidase (HRP), The latter is detected using luminol solution. Active HRP enzyme catalyzes a reaction with luminol, which thereby emits light which is detected on audoradiography film.

2.5.1 GEL PREPARATION

Two different gels were polymerized on top of each other: on top a stacking gel with a low amount of polyacrylamide for stacking the proteins before separation, and below a running gel with a higher amount of polyacrylamide for separation of the proteins.

Stacking gel contained 5% polyacrylamide solution (from stock solution containing 30% Acrylamide/ Bis solution, BioRad, Richmond, CA) (final concentration nof 5 % gel: 15% polyacrylamide, 25 % Tris-HCl (pH 6.8)+0.4 % SDS, 0.6% ammonium persulfate (APS), 0.2 % *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED)), and the running gel contained 10% polyacrylamide/BIS (final concentration of 10 % gel: 33% polyacrylamide, 25 % Tris-HCl (pH8.8)+0.4 % SDS, 0.3% APS, 0.1 % TEMED).

Other solutions added to polyacrylamide while making both running and stacking gel are presented in APPENDIX.

2.5.2 GEL ELECTROPHORESIS

The gel cassette was assembled and running buffer was added to the upper reservoir, The samples, dissolved in 1 % SDS, were added to sample buffer containing (final conc.) 2 % (w/w) SDS, 10 % (w/v) glycerol, 50 mM Tris/HCl (pH 6.8), 0.25 % (w/w) bromophenol blue, 0,1 M dithiothreitol), and equal amounts of total protein (10 µg/lane, determined by procedure described in 2.3) were applied to the individual lanes in the stacking gel.

We used protein standard (Precision Plus Protein Dual Color Standards, BioRad, Richmond, CA), as a reference to determine the molecular weight of proteins of interest identified by antibody probes.

Gel electrophoresis was run at 120 volts in a running buffer (final concentration 0.64 M Glycine, 0.08 M Tris), for approximately 80-90 minutes, and stopped when the bromophenol blue dye front reached the bottom of the gel.

2.5.3 MEMBRANE TRANSFER

After gel electrophoresis, transfer of proteins from polyacrylamide gel to a nitrocellulose membrane (0.2 μm pore size, Bio-Rad Laboratories, Hercules, CA, USA) was accomplished by electroblotting. In this procedure, a sandwich of gel and solid support membrane is compressed in a cassette and immersed in buffer between two parallel electrodes. A current is passed at right angles to the gel, which causes the separated proteins to electrophorese out of the gel and onto the solid support membrane. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. This membrane is called "blot".

Sponges and filters, used in membrane transfer, were soaked in Towbin buffer (final concentration 0.13 M Tris and 0.4 M Glycine), and the transfer chambers were then assembled in the following order

(-) Black-plate – sponge – filter – gel – membrane – filter – sponge – transparent plate(+)

(-) and (+) indicate anode and cathode respectively, as shown in figure 2.1. Proteins travelled from anode to cathode.

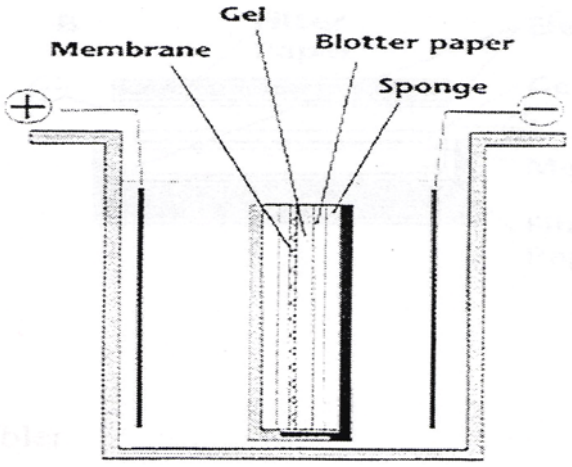


Figure 2.1 Basic principle for membrane transfer

Protein transfer was run at 21 volts overnight in Towbin buffer. After the transfer was finished, 0.2 % Ponceau S was used (Salinovich and Montelaro, 1986) to control for efficient transfer of total proteins, by coloring proteins on the membrane. Ponceau S was then washed away with Tris-buffered saline solution with 0.05% Tween 20 (TBS-Tween) (final concentration 0.067M Tris, 0.46 M NaCl and 0.05 % Tween 20).

2.5.4 BLOCKING NON SPECIFIC BINDING

Since the membrane has been chosen for its ability to bind protein, steps must be taken to prevent non-specific protein interactions between it and the antibody used for detection of the target protein. Blocking of non-specific binding was achieved by placing the membrane in 5% non-fat dry milk in TBS-Tween, for 80-90 minutes at room temperature with gentle agitation. After the blocking step, the membrane was rinsed twice in TBS-Tween.

2.5.5 ADDITION OF THE PRIMARY AND SECONDARY ANTIBODY

Primary antibody was diluted in TBS-Tween according to the optimal dilution concentration of the antibody. The blots were incubated in primary antibody for 2 hours at room temperature, with gentle agitation or left overnight at temperature of 4^{o}C. Following incubation in primary antibody the blots were washed 6 x 10 minutes, in TBS-Tween., before addition of secondary antibody.

Secondary antibody was diluted in TBS-Tween, and blots were incubated for 60 minutes at room temperature with gentle agitation. Membrane was then washed 6 x 10 minutes in TBS-Tween.

2.5.6 DETECTION

Secondary antibodies used in this assay were conjugated to enzyme HRP. This enabled chemiluminescent detection reagent to emit light by being oxidized by this enzyme, and producing intense light emission, as shown in figure 2.2. In this study, the "Amersham ECL plus ^{TM} Western Blotting Detection reagent" was used, where acridinium ester, a chemiluminescent reagent, becomes oxidized. Immediately following oxidation, the acridinium ester is in an excited state which then decays to ground state via light emitting pathway.

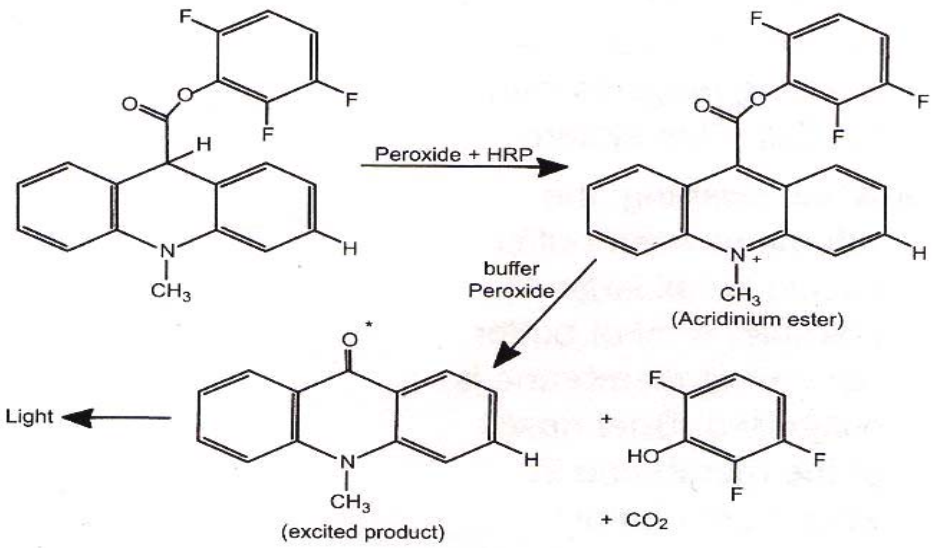


Figure 2.2 Basic principle for chemiluminescence

After the incubation in secondary antibody, blots were subsequently washed and incubated with the ECL plus ^{TM}. The signals were visualized on Hyperfilm MP (Amersham), and scanned in a desktop scanner (Scan Jet 3 c, Hewlett-Packard, Houston, TX, USA) at 600 dpi.

2.6 RECEPTOR BINDING ASSAY

2.6.1 FUNDAMENTALS OF RECEPTOR BINDING ASSAY

Radioligand binding analysis is a method used for studying receptors. There are three major types of experiments: saturation, kinetic, and inhibition. In this study, the experiment of interest was saturation binding experiment. .

For binding sites to represent functional receptors the binding needs to be specific; which means that it has to be replaceable by relevant unlabelled ligand in a relatively low concentration range. All ligands, both agonist and antagonists, also have to inhibit the binding of the radioligand to the similar level of non-displaceable binding.

2.6.2 DEFINITION OF SPECIFIC BINDING

TOTAL BINDING

Total binding is the complete quantity of the radioactivity of sample. Total binding includes both specific binding of the radioactive ligand bound to receptor of interest, and nonspecific binding to any other component.

NONSPECIFIC BINDING

Nonspecific binding includes binding of the radioligand to other receptor sites, to glass fiber filters, adsorption to the tissue, and dissolution in the membrane lipids. In carrying out nonspecific binding, appropriate excess of unlabeled drug is applied (e.g. 100 times higher than the IC_{50}) to block fully the receptors of interest (Bylund and Toews, 1993).

SPECIFIC BINDING

Specific binding can be defined as binding to the receptor of interest. Specific binding is calculated as the difference between the total binding and non-specific binding (Bylund and Toews, 1993).

2.6.3 TISSUE PREPARATION

Equipment and buffers were kept on ice during the tissue preparation. Centrifugations were carried out at $4^{o}C$, to avoid loss of binding activity.Membrane preparation was made as previously published (Kontur *et al.*, 1994) with minor adjustments.

Male rats (WKY/SHR) were anesthetized with carbon dioxide. Following decapitation, the brain was rapidly removed and placed on ice, where 10 WKY and 10 SHR rats were two months of age, and 15 WKY and 15 SHR rats were one months of age. The striatum was dissected from 34 rats, while cerebrum was dissected from 16 rats. Brains were divided by a sagittal cut, and hemibrains were obtained from 10 rats, while whole cerebrum was removed from 6 rats. Due to a small volume, two and two striata of WKY and SHR rats respectively, were combined from a total of 24 rats.

After dissection, brain samples were washed in ice-cold 50 mM Tris-HCl (pH 7.4) buffer to remove hair and blood, and then homogenized in 15 volumes 50 mM Tris-HCl buffer for 30 seconds with a glass-Teflon homogenizer.

This homogenate was centrifuged at 3500 rpm for 10 minutes. Supernatant was collected and centrifuged at 13500 rpm for 20 minutes, and the pellet was rehomogenized in 10 volumes Tris-HCl buffer for 15 seconds. In order to remove endogenous ligands, the membranes were incubated for 30 minutes at 37^{o}C followed by another centrifugation for 20 minutes at 13 500 rpm, after which the pellet was rehomogenized in 10 volumes buffer (50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$). Following rehomogenization, the membrane suspension was frozen in liquid nitrogen, and stored at -80^{o}C.

2.6.4 LIGANDS AND THE EXPERIMENTAL CONDITIONS FOR RECEPTOR BINDING ASSAY

For the analysis of D_1 antagonist binding, previously published method as described by (Kontur *et al.*, 1994; von Euler, 1991) were used, with minor adjustments.

The selected radioligand in this study was the D1-antagonist [N-methyl-^{3}H] SCH23390 (Amersham Biosciences) (figure 2.3) (Kontur *et al.*, 1994; von Euler, 1991)

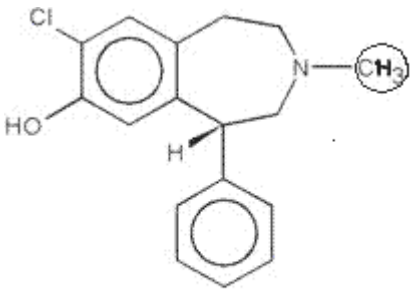


Figure 2.3 Structural formula: [N-methyl-^{3}H] SCH23390. The bold hydrogen atoms (**H**) in the circle represent the ^{3}H in the structural formula.$CH_2[^{3}H]$

The non-specific binding was defined as the binding in the presence of SCH23390 hydrochloride.

To find a suitable amount of membrane homogenate and radioactive ligand concentration to be used in receptor assay, different concentrations and volumes of the membrane homogenate were tested.

TEST 1:

Different concentrations of the radioactive ligand [^{3}H] SCH 23390 (0,5nM, 1nM, 2 nm, 5 nM, 10 nM) were tested, using the same method as indicated in receptor binding assay below. 200 µl membrane suspension from one sample was used.

TEST 2:

Different amounts of membrane suspension were used (100µl, 200µl and 300µl), with two different radioactive ligand concentration (1 nM, 2 nM). Rest of the method was followed as indicated in receptor binding assay below.

2.6.5 RECEPTOR BINDING ASSAY

Assay tubes containing 150-300 µl of either cerebrum homogenate, or 100µl of striatal homogenate, with Tris-HCl buffer and ketanserine (100 nM), were preincubated with non-specific SCH 23990 (0.5-1 µM), for 10 minutes at 25^{o}C. After the preincubation 50 µl radioactive ligand [^{3}H] SCH 23390 was added, and the incubation continued for 30 minutes at 25 ^{o}C. The incubation was terminated by rapid filtration followed by three washings with 5 ml of ice-cold 50mM Tris-HCl buffer through GF/C-filters (25 mm glassmicrofiber filter, Wathman ® , England) using a filtering box. Filters were presoaked for 20-60 minutes in 0.5% polyethylenimine to decrease nonspecific binding to the filter. Filters were counted for radioactivity by liquid scintillation spectrometry (Packard 1900 TR) in 4 ml Filter Count Scintillation Fluid (Packard Instrument Co.).

2.7 STATISTICS

Results were represented as mean ± S.E.M. The significance of differences in levels of proteins between synapsin DKO and WT mice or WKY and SHR rats, as well us differences in amounts of D_1 receptors in WKY and SHR rats were assessed on raw data by Student's t-test. Differences were considered statistically significant when $P<0.05$

3. RESULTS

This study consists of two parts. In the first part the levels of vesicular proteins in different brain regions of synapsin I/II DKO mice were examined. In the second part studies on the dopaminergic system in an animal model for ADHD were done.

3.1 THE LEVEL OF VESICULAR PROTEINS IN THREE BRAIN REGIONS OF SYNAPSIN I/II DKO MICE

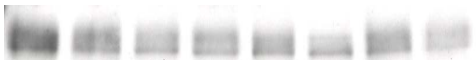
A reduction in the levels of several transporter proteins in forebrain of mice devoid of synapsin I and II, such as VGLUT-1, VGLUT-2 and VGAT, has been described by our group (Bogen *et al.,* 2006). In this study, we have focused on the cholinergic nervous system of synapsin I/II DKO mice, studying the levels of vesicular acetylcholine transporter both in the central and peripheral nervous system. It was of interest to find whether this phenomenon could be assessed on three different cholinergic regions, namely neostriatum, cortex and pons medulla. Further, the amount of transporter protein for acetylcholine, VAChT and GABA enzyme, GAD65 were also examined, to see whether the amounts of proteins are altered in these brain sections. Western blotting, as described in chapter 2.5, was used to detect the amount of the different proteins in the following studies.

In addition, the level of different vesicular proteins found in neostriatum, cortex and pons medulla in WT mice, was compared to the level found in cerebrum in WT mice.

3.1.1 VESICULAR ACETYLCHOLINE TRANSPORTER

The vesicular acetylcholine transporter, VAChT, was examined in neostriatum, cortex, pons medulla and cerebrum, in WT and synapsin I/II DKO mice. The level of VAChT was decreased by approximately 20% in neostriatum in synapsin DKO mice, as shown in table 3.1. In contrast, no significant difference was found in the other brain areas studied.

Table 3.1: The level of VAChT in neostriatum, cortex, pons medulla and cerebrum in synapsin I/II DKO mice were quantified by Western blotting. The molecular mass, in kilo Dalton (kDA), is indicated to the left of the immunoblot. Results are expressed as percent of mean levels in WT. Data are shown as mean ± SEM from 4-7 animals of each genotype. The immunoblot shown is representative of 4-7 experiments. *** $p < 0.005$ by paired Student's *t*-test.

Primary antibody	Neostriatum	Cortex	Pons Medulla	Cerebrum	Molecular mass (kDa)	Neostriatum WT	Neostriatum DKO	Cortex WT	Cortex DKO	Pons Medulla WT	Pons Medulla DKO	Cerebrum WT	Cerebrum DKO
VAChT	77 ± 2 ***	94 ± 4	84 ± 7	101 ± 4	75- 50-								

3.1.2 VESICULAR GLUTAMATE TRANSPORTER

The levels of VGLUT-1, VGLUT-2 and VGLUT-3 were measured in neostriatum, cortex, pons medulla as well as in cerebrum, in WT and synapsin I/II DKO mice. As shown in table 3.2 a 40% reduction in VGLUT-1, per total mg protein was found in cerebrum from synapsin I/II DKO mice. This compares well with the reduction per mg protein in both cortex and neostriatum, with approximately 30 and 40% decrease, respectively. The level of VGLUT-1 in pons medulla was too weak to be detected, relatively to the other brain areas studied.

The levels of VGLUT-2 in cerebrum from synapsin DKO mice were decreased to 66% of WT levels. This finding compares well with the approximate 50% reduction per total mg protein in neostriatum, and by the 40% decrease in cortex and pons medulla, respectively.

In contrast to the other two glutamate transporters, the level of VGLUT-3 per mg protein in cerebrum, neostriatum, and cortex and pons medulla was unchanged, as shown in table 3.2.

Table 3.2: Levels of VGLUT-1, VGLUT-2 and VGLUT-3 in neostriatum, cortex, pons medulla and cerebrum in synapsin I/II DKO mice were quantified by Western blotting. The molecular mass, in kilo Dalton (kDA), is indicated to the left of the immunoblot. Results are expressed as percent of mean levels in WT. Data are shown as mean ± SEM of 5-9 animals of each genotype. The immunoblots shown are representative from 5-9 experiments. * $p < 0.05$, *** $p < 0.001$ by paired Student's *t*-test.

Primary antibody	Neostriatum	Cortex	Pons Medulla	Cerebrum	Molecular mass (kDa)	Neostriatum WT	Neostriatum DKO	Cortex WT	Cortex DKO	Pons Medulla WT	Pons Medulla DKO	Cerebrum WT	Cerebrum DKO
VGLUT-1	63 ± 4 ***	70 ± 3*	Not detectable	61 ± 6*	75- 50-								
VGLUT-2	52 ± 6 *	63 ± 4 *	63± 6 *	66 ± 3 *	75- 50-								
VGLUT-3	90 ± 5	101 ± 7	86 ± 14	110 ± 14	75- 50-								

3.1.3 VESICULAR GABA TRANSPORTER

The vesicular GABA-transporter, VGAT, was examined in neostriatum, cortex, pons medulla and cerebrum, in WT and synapsin I/II DKO mice. The level of VGAT was decreased to 61% in neostriatum, 76% in cortex, 77% in pons medulla and compared well with the findings in cerebrum.

Table 3.3: The level of VGAT in neostriatum, cortex, pons medulla and cerebrum in synapsin I/II DKO mice were quantified by Western blotting. The molecular mass, in kilo Dalton (kDA), is indicated to the left of the immunoblot. Results are expressed as percent of mean levels in WT. Data are shown as mean ± SEM from 4-6 animals of each genotype. The immunoblot shown is representative of 3-6 experiments. * $p < 0.05$ by paired Student's *t*-test.

Primary antibody	Neostriatum	Cortex	Pons Medulla	Cerebrum	Molecular mass (kDa)	Neostriatum WT	Neostriatum DKO	Cortex WT	Cortex DKO	Pons Medulla WT	Pons Medulla DKO	Cerebrum WT	Cerebrum DKO
VGAT	61 ± 7*	76 ± 6*	77 ± 3*	74± 4 *	75- 50-								

3.1.4 SYNAPTOPHYSIN

Synaptophysin was used as a vesicular marker to measure the total level of synaptic vesicles. This was done both in the central and peripheral nervous system. The level of synaptophysin was measured in neostriatum, cortex, pons medulla as well as in cerebrum, in WT and synapsin I/II DKO mice. Table 3.4 shows a decrease by 66% of synaptophysin per mg protein in cerebrum from synapsin I/II DKO mice. This compares well with the reduction per total mg protein in neostriatum, cortex and pons medulla, where a decrease by 56%, 41% and 59% was detected, respectively.

Table 3.4: The level of synaptophysin in neostriatum, cortex, pons medulla and cerebrum in synapsin I/II DKO mice were quantified by Western blotting. The molecular mass, in kilo Dalton (kDA), is indicated to the left of the immunoblot. Results are expressed as percent of mean levels in WT. Data are shown as mean ± SEM from 4-7 animals of each genotype. The immunoblot shown are representative of 4-7 experiments. **p < 0.01 *** p < 0.005 by paired Student's *t*-test.

Primary antibody	Neostriatum	Cortex	Pons Medulla	Cerebrum	Molecular mass (kDa)	Neostriatum WT DKO	Cortex WT DKO	Pons Medulla WT DKO	Cerebrum WT DKO
synaptophysin	44 ± 8***	59 ± 5***	41 ± 4***	34 ± 7**	50- 37-				

3.1.5 GLUTAMIC ACID DECARBOXYLASE

GAD65 is an enzyme responsible for generation of GABA. GAD65 was examined in the neostriatum, cortex, pons medulla and cerebrum in WT and synapsin I/II DKO mice. Two bands were detected between 50 and 75 kDa, as shown in table 3.5, and both signals were quantified. No difference was found in the level of GAD65 in WT and mice devoid of synapsin I and II. Although a decrease by 20% was found in pons medulla, this was found not to be statistically significant (p-value 0.052).

Table 3.5: The level of GAD65 in neostriatum, cortex, pons medulla and cerebrum in synapsin I/II DKO mice were quantified by Western blotting. The molecular mass, in kilo Dalton (kDA), is indicated to the left of the immunoblot. Results are expressed as percent of mean levels in WT. Data are shown as mean ± SEM from 4 animals of each genotype. The immunoblot shown is representative of 4 experiments.

Primary antibody	Neostriatum	Cortex	Pons Medulla	Cerebrum	Molecular mass (kDa)	Neostriatum WT	Neostriatum DKO	Cortex WT	Cortex DKO	Pons Medulla WT	Pons Medulla DKO	Cerebrum WT	Cerebrum DKO
GAD65	92 ± 4	99 ± 5	78 ± 4	95 ± 12	75- 50-								

3.1.6 THE LEVELS OF CHOLINERGIC VESICLES IN PHERIPHERAL NEURONS IN SYNAPSIN I/II DKO

As described in chapter 2.3.3, different procedures were used for homogenate preparation of diaphragm and tongue, making it suitable for detecting VAChT by Western blotting.

Procedure 1 was a suitable method for making homogenate of diaphragm and tongue, and hereby for detecting by Western blotting, as shown by films in table 3.6. In contrast to previous blot of unclear VAChT in CNS, two bands were detected in the peripheral nervous tissue, having approximately 70 kDa molecular mass, as shown in table 3.6. Therefore both of the signals were quantified. A reduction with approximately 15 % was seen in diaphragm in mice devoid of synapsin I and II. No difference was found in the amount of VAChT in tongue. Due to few parallels, statistics were not calculated here.

Table 3.6: The level of VAChT in diaphragm and tongue in synapsin I/II DKO mice were quantified by Western blotting on fractions made by procedure 1. The molecular mass, in kilo Dalton (kDA), is indicated to the left of the immunoblot. Results are expressed as percent of mean levels in WT (WT) animals. Data are shown as mean ± SEM from 3-4 animals of each genotype. The immunoblots shown are representative of 1-3 experiments.

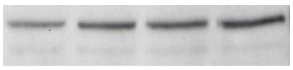
Primary Antibody VAChT		Molecular mass (kDa)	WT[4] DKO WT DKO WT DKO WT[5] DKO
Diaphragm	85 ± 7	75- 50-	
Tongue	94 ± 1	75- 50-	

In addition to procedure 1, procedure 5 gave suitable homogenates of both diaphragm and tongue, for detecting VAChT by Western blotting as indicated by the films shown in table xxx. In contrast to the samples prepared by procedure 1, a single, distinct band at approximately 70 kDa was detected. Table 3.7 indicates that the amount of VAChT in diaphragm was not statistically different in DKO mice compared to WT mice. There was an increase by approximately 11% in the tongue samples in synapsin DKO, compared to WT. Due to few parallels, statistics were not calculated here.

[4] Lane 1 of diaphragm was not quantified due to unclear signal

[5] Lane 7 of both diaphragm and tongue was not quantified due to unclear signals

Table 3.7: The level of VAChT in diaphragm and tongue in synapsin I/II DKO mice were quantified by Western blotting, on fractions made by procedure 5. The molecular mass, in kilo Dalton (kDA), is indicated to the left of the immunoblot.Results are expressed as percent of mean levels in WT (WT) animals. Data are shown as mean ± SEM from 2-3 animals of each genotype. The immunoblots shown are representative of 1-2 experiments.

Primary Antibody VAChT		Molecular mass (kDa)	WT DKO WT DKO
Diaphragm	99 ± 6	75- 50-	
Tongue	111 ± 0.13	75- 50-	

Although the same antibody was used on samples prepared by procedure 1 and procedure 5, two bands were found using procedure 1, in contrast to a single band when using samples prepared by procedure 5. The manufacturer of this VAChT antibody claims that this is a common phenomenon for large glycoproteins, e.g. VAChT, resulting in various signals. This can be due to the other proteins interacting with this vesicular transporter, e.g. myosin and actin (personal announcement by Henrik Martens from the manufacturer SYSY,).

Procedures 2-4, described in chapter 2.3.3, were not suitable methods for sample preparation for detection of the amount of VAChT in diaphragm and tongue by western blotting (films and data not shown).

I addition to VAChT, different procedures for sample preparation were tested to detect the amount of synaptophysin in the peripheral nervous system, as described in chapter 2.3.3. None of the procedures tested were suitable for detecting synaptophysin in diaphragm and tongue at the expected molecular weight (films and data not shown).

3.2 RELATIVE LEVELS OF DIFFERENT VESICULAR PROTEINS IN NEOSTRIATUM, CORTEX AND PONS MEDULLA COMPARED TO LEVELS IN THE CEREBRUM

The levels of different vesicular proteins in neostriatum, cortex and pons medulla in WT mice were compared to the levels found in total forebrain. The following comparison between levels in different brain regions and levels in total brain, are performed in WT animals using crude date from Western blotting experiments described in chapter 3.1.

Table 3.8: Relative levels of different vesicular transporters, synaptophysin and GAD65 in the neostriatum, cortex and pons medulla, compared to average levels in total cerebrum in WT mice. Results shown are expressed as percent of mean levels in cerebrum. Data are shown as means ± SEM from a total of 3-7 experiments, using crude data from experiments described in chapter 3.1. * $p < 0.05$ by paired Student's *t*-test.

Primary antibody	Cerebrum	Neostriatum	Cortex	Pons Medulla
VGLUT-1	100	92 ± 9	116 ± 13	No signal detected
VGLUT-2	100	90 ± 9	77 ± 6	145 ± 9
VGLUT-3	100	285 ± 35	125 ± 18	73 ± 21
VGAT	100	80 ± 6	87 ± 17	106 ± 25
VAChT	100	182 ± 15	103 ± 10	72 ± 6
Synaptophysin	100	129 ± 30	120 ± 21	46 ± 6 *
GAD65	100	85 ± 13	89 ± 13	110 ± 13

No signal of VGLUT-1 was detected in pons medulla related to the cerebrum in WT. Although found statistically non significant, a 45% higher amount of VGLUT-2 was seen in pons medulla related to the cerebrum in WT. The level of VGLUT-3 was almost a 3 fold higher in neostriatum compared to cerebrum, but this was found non significant.

No significant differences were found in the levels of VGAT in cortex and pons medulla in WT mice, compared to the level in cerebrum, as shown in table 3.8. The level of VGAT in neostriatum was 20% lower in neostriatum compared to the level in cerebrum.

The level of VAChT in the neostriatum was 1.8-fold higher compared to cerebrum in WT mice. This difference in VAChT level was however not statistically significant. In contrast to neostriatum, the level of VAChT in pons medulla was 28% lower compared to cerebrum, albeit this was also found no significant.

The level of synaptophysin was 50% lower in pons medulla than the level found in cerebrum. This difference was found statistically significant. In contrast, the levels of synaptophysin in neostriatum and cortex were slightly higher compared to the average level in total cerebrum.

As shown in table 3.8 the levels of GAD65 were found to be evenly distributed in striatum, cortex and pons-medulla, as compared to the levels in total forebrain.

3.3 STUDIES ON THE DOPAMINERGIC SYSTEM IN AN ANIMAL MODEL FOR ADHD

SHR is the only animal model that has been shown to demonstrate all of the behavioural characteristics of ADHD, namely hyperactivity, impulsivity and problems with sustained attention (Sagvolden, 2000; Sagvolden et al., 1992). In chapter 3.3.1 and 3.3.2 studies of D_1/D_5 receptor binding in SHR compared to control rats are described. In chapter 3.3.3, the levels of different proteins possibly involved in dopaminergic dysfunction are compared in SHR and WKY rats.

3.3.1 D_1/D_5 RECEPTOR BINDING IN SHR

Different tests were performed to find a suitable amount of the radioactive ligand concentration and amount of membrane homogenate to be used in D_1/D_5 receptor binding, as described in chapter 2.6.4

3.3.1.1 Method testing

TEST 1:

[^{3}H] SCH23390 binds selectively to dopamine D_1-like receptors (D_1 and D_5), and was added in increasing concentrations (0.5-10 nM). Non-specific binding was measured by addition of unlabelled SCH23390 (final concentration 1 µM). Binding was highest at concentrations between 0.5 and 2 nM.

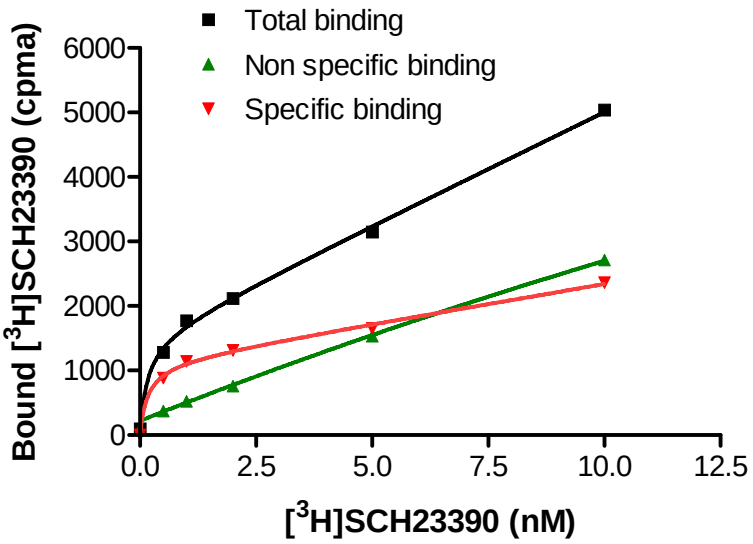


Figure 3.1: Binding of [^{3}H] SCH23390 in cerebrum homogenate with increasing ligand concentrations (0.5-10 nM) alone or in combination with 1 µM SCH23390. Specific binding was calculated as the difference between the total binding and non specific binding. Data shown represents two independent experiments.

TEST 2 :

As shown in Figure 3.1 the highest degree of specific binding was found with ligand concentrations ranging from 0.5-2 nM. Due to the small difference between specific and non specific binding for 1 and 2 nM, these concentrations were tested with increasing amount of membrane homogenate (figure 3.2)

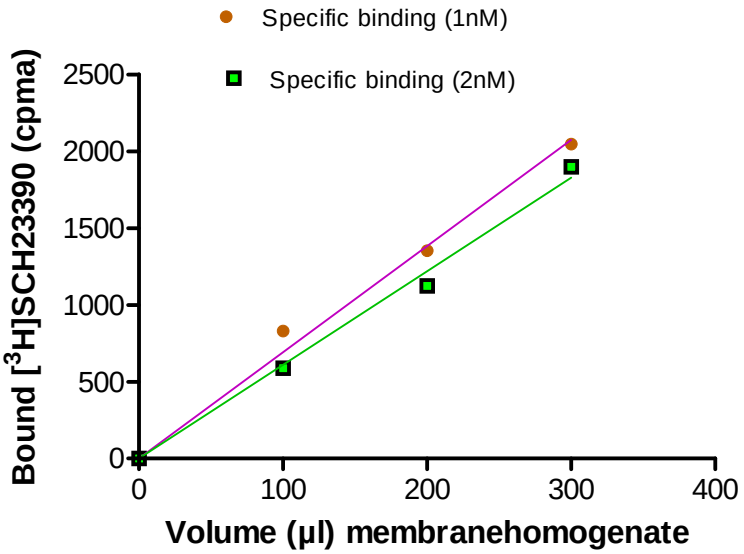


Figure 3.2: Different amounts of membrane homogenate were incubated with two concentrations of [^{3}H] SCH23390 (1 nM and 2 nM), either alone (total binding) or in combination with 1 µM SCH23390 (non specific binding). Specific binding was calculated as the difference between the total binding and non-specific binding.

3.3.2 DOPAMINE D_1- LIKE RECEPTOR IN CEREBRUM AND STRIATUM

Binding to D_1-like receptors (D_1 and D_5) was significantly increased by approximately 20% in SHR rats compared to WKY rats, using 0.5 nM [^{3}H] SCH23390. A similar result was found for 1 nM, but this was however not significant due to a larger data variation, or few replicates.

Table 3.9: Specific binding of [^{3}H] SCH23390 to dopamine D_1-like receptors measured in cerebrum. Results are expressed as percent levels of WKY animals per mg protein. Data are represented as mean ± S.E.M of 6-7 animals of each genotype. * $p < 0.05$ by paired Student's *t*-test. The control levels for ligand binding was 0.72 (0.5 nM) and 0.83 (1.0 nM) pmol/min/µg.

% specific binding to D_1 receptor in SHR rats in proportion to the control WKY			
concentration of the radioactive ligand [^{3}H] SCH23390	**% specific binding**	**p-value**	**pmol/min/µg**
0.5 nM	121 ± 6 *	0.030	WKY: 0.72
1.0 nM	118 ± 7	0.061	WKY: 1.43

A non significant reduction was seen in [^{3}H] SCH23390 binding to D_1-like receptors (D_1 and D_5) in neostriatum membrane homogenate isolated from SHR compared to WKY.

Table 3.10: Specific binding of [^{3}H] SCH23390 to dopamine D_1–like receptors measured at 1 nM in neostriatum being combined from two animals for 6 experiments, or not for 5 experiments. The data are correlated for protein, control level for binding of ligand was 1.9 pmol/min/µg. Results are expressed as percent level of WKY animals (mean ± S.E.M)of 11 animals of each genotype. The specific activity of the radioactive ligand bound in SHR and WKY animals are also expressed in pmol/min/µg

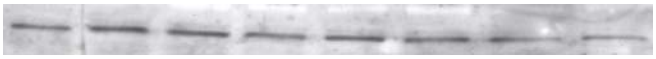
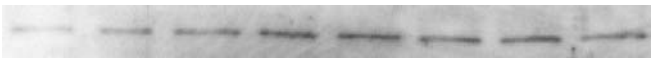
% specific binding to D_1 receptor in SHR rats in proportion to control WKY			
concentration of the radioactive ligand [^{3}H] SCH23390	**% specific binding**	**p-value**	**pmol/min/µg**
1.0 nM	93 ± 12	0.675	WKY: 1.9

3.3.3 CALCYON, COMT AND NMDA-RECEPTOR SUBUNITS

SHR was used in this study, to compare the levels of two proteins possibly involved in dopaminergic dysfunction, namely calcyon and COMT, with the levels in WKY rats. Further, the levels NMDA-Receptors were compared in SHR and WKY rats. Western blotting, as described in chapter 2.5, was used in the following studies.

The amount of the D_1-dopamine receptor interacting protein, calcyon, was examined in the neostriatum and cerebrum from SHR and compared to the levels in WKY rats. No difference in the amount of calcyon per mg protein was found in SHR rats compared to WKY in the neostriatum, nor in the cerebrum (table 3.11).

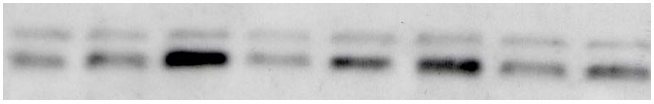
Table 3.11: Levels of calcyon in SHR rats in neostriatum and cerebrum were determined by Western blotting. Results are expressed as percent of mean levels in WKY animals. Data shown are expressed as mean ± SEM of 4 animals of each genotype. Immunoblots shown are representative of 4 experiments.

Primary Antibody Calcyon		Molecular mass (kDa)	WKY SHR WKY SHR WKY SHR WKY SHR
Neostriatum	104 ± 7	50- 37-	
Cerebrum	111 ± 6	50- 37-	

COMT is an enzyme responsible for inactivation of catecholamines, e.g. dopamine. The amount of COMT was examined in the neostriatum and cerebrum from SHR and compared to the levels of proteins in WKY rats.

Two bands were detected using the COMT antibody. The lower signal, represented a 24 kDa soluble COMT, and the upper signal at 28 kDa represented membrane bound COMT. Both of the signals were quantified, since total COMT was of our interest. As shown in table 3.12, no significant difference in the level of COMT was found in neostriatum, nor in cerebrum in SHR rats, compared to WKY rats. The level of COMT per mg protein was the same in WKY and SHR. The signals of COMT in this study showed great variations, as shown in table 3.12.

Table 3.12: Levels of COMT in SHR rats in neostriatum and cerebrum were determined by Western blotting. Results are expressed as percent of mean levels in WKY animals. Data shown are expressed as mean ± SEM of 5 animals of each genotype. Immunoblots shown are representative of 5 experiments.

Primary Antibody COMT		Molecular mass (kDa)	WKY SHR WKY[6] SHR WKY SHR WKY SHR
Neostriatum	93 ± 2	25- 20-	
Cerebrum	105 ± 2	25- 20-	

There is an interaction between NMDA receptors and dopamine D_1-like receptors (D_1 and D_5) (Cepeda and Levine, 2006; Lee and Liu, 2004). Since dopaminergic dysfunction is reported in SHR (Davids *et al.*, 2003; Oades *et al.*, 2005; Russell, 2003; Sagvolden *et al.*, 2005), the NMDA-1 receptor subunits, required for NMDA receptor activity, was examined in neostriatum and cerebrum of SHR and compared to WKY animals.

The NMDA-1 receptor subunit-antibody generated the one band as shown in table 3.13. The amount of NDMA-R1 subunit detected in SHR were nearly identical to the amount detected in WKY rats, in both neostriatum and cerebrum, indicating that there is no difference in SHR rats in expression of this receptor subunit protein

[6] Lane 3 of neostriatum, WKY was not quantified due to a contaminated sample

Table 3.13: Levels of NMDA-1 receptor subunit in SHR rats in neostriatum and cerebrum were determined by Western blotting. Results are expressed as percent of mean levels in WKY animals. Data shown are expressed as mean ± SEM of 5 animals of each genotype. Immunoblots shown are representative of 5 experiments.

Primary Antibody NMDA-1		Molecular mass (kDa)	WKY SHR WKY [7] SHR WKY SHR WKY SHR
Neostriatum	95 ± 5	150- 100-	
Cerebrum	98 ± 8	150- 100-	

The levels of the NMDA-2A/B receptor subunit was quantified in neostriatum and cerebrum of SHR and compared to the levels in WKY animals. The amount of NMDA-2A/B subunit of SHR compared to their control WKY, was the same in both neostriatum and cerebrum.

Table 3.14: Levels of NMDA-2A/B subunit in SHR in neostriatum, and cerebrum, were determined by Western blotting. Results are expressed as percent of mean levels in WKY animals. Data shown are expressed as mean ± SEM of 3-5 animals of each genotype. Immunoblots shown are representative of 3-5 experiments.

Primary Antibody NMDA-2		Molecular mass (kDa)	WKY SHR WKY SHR WKY SHR WKY SHR
Striatum	101 ± 6	250- 150-	
Cerebrum	106 ± 3	250- 150-	

[7] Lane 3 of cerebrum, WKY, was not quantified due to sample contaminations.

4. DISCUSSION

In this study, two different animal models with implications for neurological disorders have been examined. In the first part, the aim was to observe the consequences of deletion of synapsin I and II for different vesicular subgroups of vesicles in different brain regions. The effects on the cholinergic vesicles were of our main interest since the consequences of absence of synapsin I and II for this particular subgroup of vesicles have previously not been studied.

In the second part, the aim was to measure the levels of proteins involved in the dopaminergic system in spontaneous hypertensive rats (SHR), serving as an animal models for the "attention deficity hyperactivity disorder" (ADHD). Since many dopaminergic effects are mediated through the interaction with glutamatergic neurotransmission, levels of different NMDA-receptor subunits were also examined.

4.1 THE LEVEL OF VESICULAR PROTEINS IN SYNAPSIN I/II DOUBLE KNOCKOUT MICE

This study is based on the results of Bogen *et al*., (2006), with the main focus on the effects on cholinergic vesicles when synapsin I and II are absent. VAChT was used as a marker for cholinergic vesicles and was quantified in three different cholinergic centra, namely the neostriatum, cortex and pons medulla. This is due to that neostriatum contains cholinergic interneurons, cortex have long cholinergic fibers originating in the nucleaus basalis and pons medulla project cholinergic terminals.

4.1.1 THE LEVELS OF VESICULAR TRANSPORTERS IN SYNAPSIN I/II DOUBLE KNOCKOUT MICE IN CENTRAL NERVOUS SYSTEM

VAChT was examined in synapsin I/II DKO mice to detect whether there was a change in the levels of the transporter responsible for transporting ACh into synaptic vesicles. A decrease by 23% was found in the neostriatum of synapsin DKO mice compared to wild-type, while no difference was found in the cortex, pons medulla or whole forebrain. These results indicate that lack of synapsin I and II is of importance for the interneurons in the

striatum, while the cholinergic vesicles in cortex, pons medulla and total forebrain are unaffected.

Both VGLUT-1, VGLUT-2 and VGAT were decreased by approximately 30-50% in neostriatum, cortex and whole cerebrum of synapsin I/II DKO. The levels of VGLUT-1 in pons medulla were to low to be quantified, while the levels of VGLUT-2 and VGAT were decreased to 63% and 77%, respectively, in pons medulla in synapsin I/II DKO. In contrast, no decrease was found in VGLUT-3 containing vesicles in neither of the examined brain areas. The general vesicle marker, synaptophysin, was decreased by approximately 40-60% in different brain areas, indicating a 40-60% decrease in the number of synaptic vesicles in synapsin DKO mice. This compares well with a 50% decrease in number of vesicles in synapsin DKO mice obtained by electron microscopy by Rosahl *et.al*., (1995) and 40% decrease detected in the levels of different general vesicle markers by Bogen *et al*., (2006). The findings of this study on the levels of VGLUT1-3 and VGAT in neostriatum, cortex and pons medulla compare well with the previous findings in cerebrum performed by our group (Bogen *et al.*, 2006). This indicates that the decreases in the levels of specific synaptic vesicles is a general phenomena evenly distributed in forebrain, and not restricted to a specific brain region.

GAD65, an isoform of the enzyme glutamate decarboxylase, was of interest in this study, being responsible for converting glutamate to GABA. No statistical difference in the amount of GAD65 per mg protein in synapsin I/II DKO mice was found, compared to WT mice. A ~22% decrease in the level of GAD65 in pons medulla almost reached statistical significance (p-value 0.052) and may be of biological relevance. The finding that GAD65 is not reduced in the neostriatum, cortex and whole cerebrum of synapsin I/II DKO indicate that GABA synthesis in inhibitory synapses was not restrained by a reduced GAD65 activity. (Benson *et al.*, 1994) showed that GAD65 was positively immunostained in hippocampal neurons for synapsin I, suggesting that GAD65-positive varicosities that contact the somata and dendrites represent presynaptic specializations. The reason why the amount of GAD65 was not affected by the absence of synapsin I and II could be due to the fact that although GAD65 is found in membranes of synaptic vesicles, a pool of GAD65 is also found in the Golgi complex region of neurons, hereby "masking" an eventual loss of the enzyme in the vesicles (Kanaani *et al.*, 1999). In addition the enzymatic activity of GAD65 may be altered, although the levels of proteins were not changed.

4.1.2 THE LEVELS OF CHOLINERGIC VESICLES IN PERIPHERAL NEURONS IN SYNAPSIN I/II DKO

There are few publications on VAChT in the PNS. No significant decrease in the levels of VAChT was found in preparations of tounge or diaphragm from synapsin I/II DKO mice. However, none of the sample preparations obtained from tongue and diaphragm were suitable for detection of synaptophysin by Western blotting. It therefore appears possible that the amount of synaptic vesicles in preparations from skeletal muscle is insufficient to allow biochemical detection by immunoblotting. To address the question whether cholinergic vesicles in the peripheral nervous system co-localize with synapsins, methods such as confocal microscopy or electron microscopy could be employed.

4.1.3 DIFFERENCE IN THE DEPENDENCE OF SYNAPSIN I AND II FOR SPECIFIC VESICULAR TRANSPORTERS

The majority of vertebrate nerve terminals have been presumed to contain synapsin proteins (De Camilli et al., 1983; Sudhof, 2004; Walaas, Browning, and Greengard, 1988). Previous studies have reported major decreases in the number of synaptic vesicles in the reserve pool of synapsin deficient brains (Gitler *et al.*, 2004; Li *et al.*, 1995b; Rosahl *et al.*, 1995; Takei *et al.*, 1995). The findings of Bogen et al. (2006) that VGLUT-3 and VMAT2 in striatum and hippocampus do not co-localize with neither synapsin I nor II were therefore surprising. This study confirms the findings by Bogen et al. (2006), and further, have revealed another specific neurotransmitter transporter, VAChT, which is not present at reduced levels in forebrains of synapsin DKO mice.

The molecular mechanisms responsible for the decreases in synaptic vesicles and vesicular transporters in the absence of synapsin I and II, remain unclear. The possibility that synapsin proteins could modulate the synthesis of vesicular transporters appears unlikely, since mRNA levels excluded a decreased expression of the genes encoding VGLUT-1 and VGLUT-2 (Bogen et al., 2006). Synaptic vesicles are covered with synapsins and could serve some role of vesicle stabilization. Lack of synapsins could therefore induce the vesicles to undergo spontaneous degradation. This was first suggested by Rosahl *et al.*, (1995) saying that the decrease in synaptic vesicle proteins is due to a premature degradation in synapsin knockout mice. This degradation appears to occur in nerve

terminals as no increase in synaptic vesicle proteins could be detected in the cell bodies of the mutant mice (Rosahl *et.al*., 1995).

The present data indicate that synaptic vesicles have a different vulnerability to degradation, based on their distinct relation to synapsin proteins. Anatomical studies, using confocal microscopy, indicated that VGLUT-1, VGLUT-2 and VGAT were co-localized with synapsins I and/or II in the areas studied (Bogen et al., 2006). In contrast, VGLUT-3 and VMAT2, not decreased in mice lacking synapsin I and II, were not found to co-localize with synapsin I or II (Bogen et al., 2006). This indicates that VGLUT-3, VMAT2 and probably also VAChT, present in modulatory synapses, are present in synapsin I/II-negative synaptic vesicles. These specific subgroups of vesicles were not decreased in the synapsin I/II double knockout. Confocal studies of VAChT and synapsin I and II must be performed to find out whether VAChT and synapsin I/II co-localize.

We have studied the levels of VAChT in three different brain areas of synapsin DKO mice; the cholinergic long fibers in the cortex, interneurons in the neostriatum, and cholinergic terminals with origin in pons medulla. Our results indicate that the cholinergic interneurons in striatum, being decreased by 23% in synapsin DKO, are synapsin-dependent, while the cholinergic neurons in cortex and pons-medulla are not. Again, this can be approached by using e.g. confocal microscopy.

In addition, we have studied the level of VAChT and synaptophysin in the PMS. There was no reduction in VAChT in the PMS in synapsin I/II DKO, which can indicate that these synaptic vesicles are synapsin I and II independent.

4.1.3 PROTEINS IN WILD-TYPE MICE IN THREE BRAIN SECTIONS COMPARED TO CEREBRUM

The levels of different vesicular proteins found in neostriatum, cortex and pons medulla in wild-type mice, were compared to the levels found in total forebrain.

In our studies, the amount of VGLUT-1 was found to be slightly higher in cortex, compared to the level in whole cerebrum. The levels of VGLUT-1 in pons medulla were very low and not possible to quantify. Our findings correlate well with previous reports,

showing high levels of VGLUT-1 in the cortex (Fremeau *et al.*, 2001; Varoqui *et al.*, 2002), most terminals in the synaptic fields in the dentate gyrus, hippocampus, striatum, and very low levels in the pons medulla (Bellocchio *et al.*, 1998; Fremeau *et al.*, 2001).

VGLUT-2 is found in the pons medulla, brainstem, thalamus, striatum and hypothalamus, while lower levels are found in the cortex and hippocampus (Fremeau *et al.*, 2001; Herzog *et al.*, 2001; Varoqui *et al.*, 2002). In this study 45% higher and 23% lower levels of VGLUT-2 was seen in pons medulla of wild type mice compared to cerebrum.

VGLUT3 is expressed in much more restricted cell populations compared to VGLUT1 and VGLUT2 (Gras *et al.*, 2002). An even more striking finding is that VGLUT3 is found in populations of non-glutamatergic cells, such as cholinergic interneurons in the striatum and nucleus accumbens, GABAergic interneurons in the cortex, hippocampus and interpeduncular nucleus, and in serotonergic neurons of the raphe nuclei (Fremeau *et al.*, 2002; Gras *et al.*, 2002). The levels of VGLUT-3 were found to be 3-fold higher in the neostriatum compared to the level in total cerebrum.

It has previously been reported that VGAT is evenly distributed in the brain (McIntire *et al.*, 1997; Sagne *et al.*, 1997), which was in accordance with our findings. Also GAD65, predominately present in membranes and GABAergic nerve endings (Kanaani et al., 1999; Soghomonian and Martin, 1998) was found at similar levels in all the brain areas studied.

The level of VAChT was approximately two-fold in the neostriatum, and slightly lower in pons-medulla compared to the level in whole cerebrum. The level of synaptophysin was also lower in the pons medulla, possibly indicating a lower total number of nerve terminals and synaptic vesicles in this brain region. Litterature supporting these findings was not found.

4.2 STUDIES ON THE DOPAMINERGIC SYSTEM IN AN ANIMAL MODEL FOR ADHD

Increasing evidence indicates that dysfunction of dopaminergic projections in the brain may be involved in major symptoms of ADHD (Davids *et al.*, 2003; Oades *et al.*, 2005; Russell, 2003; Sagvolden *et al.*, 2005). Further support for this dopamine hypothesis has been provided by studies on the spontaneous hypertensive rat (SHR), the only animal

model that demonstrates the behavioural characteristics of ADHD, namely hyperactivity, impulsivity and problems with sustained attention (Sagvolden, 2000; Sagvolden et al., 1992).

4.2.1 THE D_1-LIKE RECEPTOR BINDING IN SHR COMPARED TO WKY

A genetic association between ADHD and changes in the dopamine D_1 - like receptor genes has been shown (Faraone and Khan, 2006; Faraone et al., 2005). Experiments have shown an impaired dopamine release in SHR (Russell *et al.*, 1998), and the density of D1-like receptors might be changed as well in the brain of SHR. However, it should be kept in mind that the SHR genotype is genetically complex, and there is no reason to expect that the phenotypical similarities between the human condition and the rat phenotype necessarily will be reflected in completely identical molecular changes.

Conflicting data have been presented regarding dopamine receptor binding in the brain of SHR. In this study, employing binding analysis with the selective dopamine D1-like receptor antagonist [^{3}H]SCH23390 (Bourne, 2001; Hyttel, 1983) on membranes from cerebrum and neostriatum from 4 weeks old prehypertensive and 8 weeks old posthypertensive SHR rats, we found that D_1–like receptor binding was increased by 20 % in cerebrum of SHR, compared to control rats. This occurred both when using 0.5 nM [^{3}H] SCH23390 and, with a slightly smaller effect, when using 1 nM of [^{3}H] SCH23390 although the latter did not reach statistical significance (p-value 0.061 by paired Student's *t*-test). In contrast, the density of D_1-like receptors was not altered in neostriatal membranes from SHR animals. These results may indicate a plausible up-regulation of $D_1/_5$-like receptors in widespread areas of the cerebrum, possibly as a consequence of reduced dopamine release in the SHR animals (Russell, 2003).

These results are compatible with other studies. Due to the fact that the major part of brain dopamine is restricted to three systems in brain, i.e., the mesocortical, mesolimbic and nigrostriatal branches, previous reports on dopamine receptor binding in brain have investigated one or more of these systems. In a study by Carey *et al.* (1998), a higher density of D-1 like receptors was seen in the core and shell of the nucleus accumbens, and the olfactory tubercle. A down-regulation was found in the globus pallidus and septum. This is in accordance with findings by Kirouac & Ganguly, (1993), which showed increased density of D1-like receptor in nucleus accumbens, septal nuclei and globus

pallidus using receptor autoradiography. Preparations from SHR showed significantly higher binding of [^{3}H] SCH23390 in nucleus accumbens and olfactory tubercle in a study reported by Kujirai *et al*. (1990). However, SHR was here compared to Sprague-Dawley rats, and whether the latter rat strain is an appropriate control strain for SHR is debatable (Linthorst *et al.*, 1993).

Other reports are more conflicting. While several reports show increases in density of D_1-like receptors in caudate putamen and nucleus accumbens in SHR, either using autoradiography (Carey et al., 1998; Kirouac and Ganguly, 1993; Kujirai et al., 1990; Watanabe et al., 1997) or striatal membrane preparations (Lim *et al.*, 1989; Lim *et al.*, 1990), other studies report no difference (Linthorst *et al.*, 1993; Watanabe *et al.*, 1989).
This discrepancy, with some studies showing no increase in striatum may be due to distinct methodological approaches being used. In addition, in the paper by Kujirai *et al.* (1990), Sprague Dawley rats were used as control rats, which may not be a proper control (see above). Moreover, genetic and functional heterogeneity may also be present in WKY animals from different suppliers (T. Sagvolden and Ø. Hvalby, personal communications).

D_1-like receptors have been reported to be functionally connected to working memory (Russell *et al.*, 1998; Sagvolden *et al.*, 2005). Working memory may have implications on delayed gradients and how reinforcers[8] work. Shorter working memory, which may be caused by hypofunctioning of dopamine, means that the time window to associate behaviour with its consequences is shorter in ADHD. Reinforcers will then have low effect due to short time window. This can explain both the hyperactivity and impulsiveness, an explanation based on the dynamic developmental theory of ADHD, where each symptom can be explained through the effect of reinforcers (Sagvolden *et al.*, 2005).

4.2.2 CALCYON AND COMT

Since dopaminergic systems have been hypothesized to be involved in the development of ADHD (Davids *et al.*, 2003; Oades *et al.*, 2005; Russell, 2003; Sagvolden *et al.*, 2005), calcyon protein, a brain-specific D_1-receptor-interacting protein involved in the Ca^{2+}-signaling of D_1/D_5, was examined in this study in both neostriatum and cerebrum of SHR. No difference in the amount of calcyon was found in SHR rats compared to WKY rats in neostriatum and cerebrum. This contrasts with preliminary findings of Sagvolden, Walaas,

[8] Reinforcement associates behaviour and consequences

Middleton and Faraone (2006, in.prep.), where an increase in the level of mRNA encoding calcyon was seen. In a study by Laurin *et al.,* (2005) the calcyon gene was implicated as a risk factor in the development of the ADHD. Although no change in the amount of calcyon protein was detected in neostriaum or in cerebrum in our study, one cannot exclude that calcyon is affected in ADHD. It is possible that Western blotting is insufficiently sensitive in order to detect small changes in proteins. A large number of replicates would then be needed in order to detect statistically significant changes.

COMT (catecholamine O-methyltransferase) is an enzyme which catalyzes the degradation of catecholamines, including dopamine It was therefore of interest in this investigation to see whether the amount of COMT is changed in SHR compared to their WKY control rats. No significant difference was found in the amount of COMT proteins in SHR rats compared their control WKY rats in neostriatum and cerebrum. Although extensive research has been done concerning the genetics of COMT and ADHD, e.g. on association between *Val*^{158} *Met* polymorphism and ADHD, only limited research has been performed on protein levels of COMT in SHR,. The Val variant is associated with a higher activity of COMT and thermal stability, and has been convincingly implicated in a poor frontal lobe function across a number of cognitive tasks (Cheuk and Wong, 2006). However, meta analysis on studies between COMT polymorphisms and ADHD concluded that no significant association was present between the most commonT gene polymorphism and ADHD (Cheuk and Wong, 2006).

4.2.3 NMDA-RECEPTORS

Dopamine effects in brain are often mediated by modulation of major excitatory neurotransmission mechanisms, at either presynaptic or postsynaptic levels. Recent studies have shown an interaction between D_1-receptors and the glutamate receptors designated NMDA-receptors in brain cells (Cepeda and Levine, 2006; Lee and Liu, 2004). This interaction between D_1 and NMDA receptors has been proposed to involve activation of second-messenger signalling cascades after receptor stimulation. However, in recent years, another paradigm has emerged; involving the direct interaction between D_1 and NMDA receptors. Since we found that the level of D_1 receptors was shown to be increased in cerebrum of SHR, one might expect changes in the amount of NMDA-R subunits as well. However, no differences was found in the levels of NMDA-1 and NMDA-2A/B in SHR compared to control WKY rats, either in neostriatum or in cerebrum.

In contrast, preliminary findings by Sagvolden *et .al.,* (2006, in prep.) on different levels of mRNA encoding these glutamate receptor subunits indicated possible differences, again emphasizing that levels and stability of mRNA may be under separate controls when compared to the steady state levels of the encoded proteins.

Reports of activity-induced changes in the density of both D_1/D_5 and NMDA receptors have been presented (Cepeda and Levine, 2006; Lee and Liu, 2004). While NMDA receptor activity may recruit D_1 receptors to the cell surface, the effect of D_1 activation on NMDA receptors density is reported to be either decreased (Lee and Liu, 2004), or increased (Cepeda and Levine, 2006). This interaction can therefore have significant implications in the generation of rational new treatments for psychiatric and neurological disorders, e.g. ADHD (Cepeda and Levine, 2006).

4.3 CONCLUSION

In the first part of this study, we focused on the three cholinergic centra in synapsin I/II DKO to see whether there is a change in VAChT in synapsin I/II DKO. In addition glutamatergic and gabaergic vesicular transporters were examined in the same brain areas of synapsin I/II DKO. Our results showed a decrease of VAChT in neostriatum, indicating that synapsin I/II is of importance for the cholinergic interneurones found in neostriatum, in contrast to the other cholinergic centra examined. In addition, the reduction in VGLUT-1-2 an VGAT, and no change in VGLUT-3 in three brain areas of synapsin I/II DKO gave support to previous findings by our group, saying that some synaptic vesicles are synapsin dependent, while others are not. To further evaluate whether cholinergic centra are synapsin I and/or II dependent, methods like confocal microscopy and electron microscopy may be used to assess this question

In the second part of this study we found an increase in the brain density of D_1-like receptors in the SHR, an animal model of ADHD, This increase might be related to the proposed hypofunctioning of dopamine in SHR, and may thus indirectly support the hypothesis of dopaminergic system dysregulation in ADHD. Otherwise, no changes were detected in the SHR when examining the protein levels of the dopaminergic markers calcyon and COMT, and the glutamate receptor form NMDA-R.

Hence, these data give only preliminary support to a possible link between dopaminergic hypofunction and ADHD aetiology.

REFERENCES

Adriani, W., Caprioli, A., Granstrem, O., Carli, M. & Laviola, G. (2003) The spontaneously hypertensive-rat as an animal model of ADHD: evidence for impulsive and non-impulsive subpopulations. *Neurosci Biobehav Rev* **27**(7), 639-51.

Arvidsson, U., Riedl, M., Elde, R. & Meister, B. (1997) Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. *J Comp Neurol* **378**(4), 454-67.

Bellocchio, E.E., Hu, H., Pohorille, A., Chan, J., Pickel, V.M. & Edwards, R.H. (1998) The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. *J Neurosci* **18**(21), 8648-59.

Benson, D.L., Watkins, F.H., Steward, O. & Banker, G. (1994) Characterization of GABAergic neurons in hippocampal cell cultures. *J Neurocytol* **23**(5), 279-95.

Bogen, I.L., Boulland, J.L., Mariussen, E., Wright, M.S., Fonnum, F., Kao, H.T. & Walaas, S.I. (2006) Absence of synapsin I and II is accompanied by decreases in vesicular transport of specific neurotransmitters. *J Neurochem* **96**(5), 1458-66.

Bourne, J.A. (2001) SCH 23390: the first selective dopamine D1-like receptor antagonist. *CNS Drug Rev* **7**(4), 399-414.

Brusa, R. (1999) Genetically modified mice in neuropharmacology. *Pharmacol Res* **39**(6), 405-19.

Bu, D.F., Erlander, M.G., Hitz, B.C., Tillakaratne, N.J., Kaufman, D.L., Wagner-McPherson, C.B., Evans, G.A. & Tobin, A.J. (1992) Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. *Proc Natl Acad Sci U S A* **89**(6), 2115-9.

Bylund, D.B. & Toews, M.L. (1993) Radioligand binding methods: practical guide and tips. *Am J Physiol* **265**(5 Pt 1), L421-9.

Carey, M.P., Diewald, L.M., Esposito, F.J., Pellicano, M.P., Gironi Carnevale, U.A., Sergeant, J.A., Papa, M. & Sadile, A.G. (1998) Differential distribution, affinity and plasticity of dopamine D-1 and D-2 receptors in the target sites of the mesolimbic system in an animal model of ADHD. *Behav Brain Res* **94**(1), 173-85.

Castellanos, F.X. & Tannock, R. (2002) Neuroscience of attention-deficit/hyperactivity disorder: the search for endophenotypes. *Nat Rev Neurosci* **3**(8), 617-28.

Cepeda, C. & Levine, M.S. (2006) Where do you think you are going? The NMDA-D1 receptor trap. *Sci STKE* **2006**(333), pe20.

Cheuk, D.K. & Wong, V. (2006) Meta-analysis of Association Between a Catechol-O-Methyltransferase Gene Polymorphism and Attention Deficit Hyperactivity Disorder. *Behav Genet*.

Cohen-Cory, S. (2002) The developing synapse: construction and modulation of synaptic structures and circuits. *Science* **298**(5594), 770-6.

Davids, E., Zhang, K., Tarazi, F.I. & Baldessarini, R.J. (2003) Animal models of attention-deficit hyperactivity disorder. *Brain Res Brain Res Rev* **42**(1), 1-21.

De Camilli, P., Harris, S.M., Jr., Huttner, W.B. & Greengard, P. (1983) Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J Cell Biol* **96**(5), 1355-73.

Faraone, S.V. & Khan, S.A. (2006) Candidate gene studies of attention-deficit/hyperactivity disorder. *J Clin Psychiatry* **67 Suppl 8**, 13-20.

Faraone, S.V., Perlis, R.H., Doyle, A.E., Smoller, J.W., Goralnick, J.J., Holmgren, M.A. & Sklar, P. (2005) Molecular genetics of attention-deficit/hyperactivity disorder. *Biol Psychiatry* **57**(11), 1313-23.

Ferreira, A. & Rapoport, M. (2002) The synapsins: beyond the regulation of neurotransmitter release. *Cell Mol Life Sci* **59**(4), 589-95.

Fremeau, R.T., Jr., Burman, J., Qureshi, T., Tran, C.H., Proctor, J., Johnson, J., Zhang, H., Sulzer, D., Copenhagen, D.R., Storm-Mathisen, J., Reimer, R.J., Chaudhry, F.A. & Edwards, R.H. (2002) The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc Natl Acad Sci U S A* **99**(22), 14488-93.

Fremeau, R.T., Jr., Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Bellocchio, E.E., Fortin, D., Storm-Mathisen, J. & Edwards, R.H. (2001) The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron* **31**(2), 247-60.

Fremeau, R.T., Jr., Voglmaier, S., Seal, R.P. & Edwards, R.H. (2004) VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci* **27**(2), 98-103.

Fykse, E.M. & Fonnum, F. (1988) Uptake of gamma-aminobutyric acid by a synaptic vesicle fraction isolated from rat brain. *J Neurochem* **50**(4), 1237-42.

Fykse, E.M. & Fonnum, F. (1996) Amino acid neurotransmission: dynamics of vesicular uptake. *Neurochem Res* **21**(9), 1053-60.

Galli-Taliadoros, L.A., Sedgwick, J.D., Wood, S.A. & Korner, H. (1995) Gene knock-out technology: a methodological overview for the interested novice. *J Immunol Methods* **181**(1), 1-15.

Gasnier, B. (2000) The loading of neurotransmitters into synaptic vesicles. *Biochimie* **82**(4), 327-37.

Gincel, D. & Shoshan-Barmatz, V. (2002) The synaptic vesicle protein synaptophysin: purification and characterization of its channel activity. *Biophys J* **83**(6), 3223-9.

Gitler, D., Takagishi, Y., Feng, J., Ren, Y., Rodriguiz, R.M., Wetsel, W.C., Greengard, P. & Augustine, G.J. (2004) Different presynaptic roles of synapsins at excitatory and inhibitory synapses. *J Neurosci* **24**(50), 11368-80.

Gras, C., Herzog, E., Bellenchi, G.C., Bernard, V., Ravassard, P., Pohl, M., Gasnier, B., Giros, B. & El Mestikawy, S. (2002) A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *J Neurosci* **22**(13), 5442-51.

Herzog, E., Bellenchi, G.C., Gras, C., Bernard, V., Ravassard, P., Bedet, C., Gasnier, B., Giros, B. & El Mestikawy, S. (2001) The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *J Neurosci* **21**(22), RC181.

Hilfiker, S., Schweizer, F.E., Kao, H.T., Czernik, A.J., Greengard, P. & Augustine, G.J. (1998) Two sites of action for synapsin domain E in regulating neurotransmitter release. *Nat Neurosci* **1**(1), 29-35.

Hosaka, M., Hammer, R.E. & Sudhof, T.C. (1999) A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. *Neuron* **24**(2), 377-87.

Hyttel, J. (1983) SCH 23390 - the first selective dopamine D-1 antagonist. *Eur J Pharmacol* **91**(1), 153-4.

Jaber, M., Robinson, S.W., Missale, C. & Caron, M.G. (1996) Dopamine receptors and brain function. *Neuropharmacology* **35**(11), 1503-19.

Jahn, R., Schiebler, W., Oiumet, C. & Greengard, P. (1985) A 38,000-dalton membrane protein(p38) presen in the synaptic vesicles. *Proc.Natl.Acad.Sci.USA* **82**, 4137-4141.

Kanaani, J., Lissin, D., Kash, S.F. & Baekkeskov, S. (1999) The hydrophilic isoform of glutamate decarboxylase, GAD67, is targeted to membranes and nerve terminals independent of dimerization with the hydrophobic membrane-anchored isoform, GAD65. *J Biol Chem* **274**(52), 37200-9.

Kao, H.T., Porton, B., Czernik, A.J., Feng, J., Yiu, G., Haring, M., Benfenati, F. & Greengard, P. (1998) A third member of the synapsin gene family. *Proc Natl Acad Sci U S A* **95**(8), 4667-72.

Kao, H.T., Porton, B., Hilfiker, S., Stefani, G., Pieribone, V.A., DeSalle, R. & Greengard, P. (1999) Molecular evolution of the synapsin gene family. *J Exp Zool* **285**(4), 360-77.

Kirouac, G.J. & Ganguly, P.K. (1993) Up-regulation of dopamine receptors in the brain of the spontaneously hypertensive rat: an autoradiographic analysis. *Neuroscience* **52**(1), 135-41.

Kontur, P.J., al-Tikriti, M., Innis, R.B. & Roth, R.H. (1994) Postmortem stability of monoamines, their metabolites, and receptor binding in rat brain regions. *J Neurochem* **62**(1), 282-90.

Kujirai, K., Przedborski, S., Kostic, V., Jackson-Lewis, V., Fahn, S. & Cadet, J.L. (1990) Autoradiography of dopamine receptors and dopamine uptake sites in the spontaneously hypertensive rat. *Brain Res Bull* **25**(5), 703-9.

Laurin, N., Misener, V.L., Crosbie, J., Ickowicz, A., Pathare, T., Roberts, W., Malone, M., Tannock, R., Schachar, R., Kennedy, J.L. & Barr, C.L. (2005) Association of the calcyon gene (DRD1IP) with attention deficit/hyperactivity disorder. *Mol Psychiatry* **10**(12), 1117-25.

Lee, F.J. & Liu, F. (2004) Direct interactions between NMDA and D1 receptors: a tale of tails. *Biochem Soc Trans* **32**(Pt 6), 1032-6.

Leube, R.E. (1994) Expression of the synaptophysin gene family is not restricted to neuronal and neuroendocrine differentiation in rat and human. *Differentiation* **56**(3), 163-71.

Lezcano, N., Mrzljak, L., Eubanks, S., Levenson, R., Goldman-Rakic, P. & Bergson, C. (2000) Dual signaling regulated by calcyon, a D1 dopamine receptor interacting protein. *Science* **287**(5458), 1660-4.

Li, L., Chin, L.S., Greengard, P., Copeland, N.G., Gilbert, D.J. & Jenkins, N.A. (1995a) Localization of the synapsin II (SYN2) gene to human chromosome 3 and mouse chromosome 6. *Genomics* **28**(2), 365-6.

Li, L., Chin, L.S., Shupliakov, O., Brodin, L., Sihra, T.S., Hvalby, O., Jensen, V., Zheng, D., McNamara, J.O., Greengard, P. & et al. (1995b) Impairment of synaptic vesicle clustering and of synaptic transmission, and increased seizure propensity, in synapsin I-deficient mice. *Proc Natl Acad Sci U S A* **92**(20), 9235-9.

Li, X., Rosahl, T.W., Sudhof, T.C. & Francke, U. (1995c) Mapping of synapsin II (SYN2) genes to human chromosome 3p and mouse chromosome 6 band F. *Cytogenet Cell Genet* **71**(3), 301-5.

Lim, D.K., Ito, Y., Hoskins, B., Rockhold, R.W. & Ho, I.K. (1989) Comparative studies of muscarinic and dopamine receptors in three strains of rat. *Eur J Pharmacol* **165**(2-3), 279-87.

Lim, D.K., Yu, Z.J., Hoskins, B., Rockhold, R.W. & Ho, I.K. (1990) Effects of acute and subacute cocaine administration on the CNS dopaminergic system in Wistar-Kyoto and spontaneously hypertensive rats: II. Dopamine receptors. *Neurochem Res* **15**(6), 621-7.

Linthorst, A.C., De Jong, W., De Boer, T. & Versteeg, D.H. (1993) Dopamine D1 and D2 receptors in the caudate nucleus of spontaneously hypertensive rats and normotensive Wistar-Kyoto rats. *Brain Res* **602**(1), 119-25.

Lucas-Meunier, E., Fossier, P., Baux, G. & Amar, M. (2003) Cholinergic modulation of the cortical neuronal network. *Pflugers Arch* **446**(1), 17-29.

Masson, J., Sagne, C., Hamon, M. & El Mestikawy, S. (1999) Neurotransmitter transporters in the central nervous system. *Pharmacol Rev* **51**(3), 439-64.

Maycox, P.R., Hell, J.W. & Jahn, R. (1990) Amino acid neurotransmission: spotlight on synaptic vesicles. *Trends Neurosci* **13**(3), 83-7.

McIntire, S.L., Reimer, R.J., Schuske, K., Edwards, R.H. & Jorgensen, E.M. (1997) Identification and characterization of the vesicular GABA transporter. *Nature* **389**(6653), 870-6.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N. & Nakanishi, S. (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature* **354**(6348), 31-7.

Oades, R.D., Sadile, A.G., Sagvolden, T., Viggiano, D., Zuddas, A., Devoto, P., Aase, H., Johansen, E.B., Ruocco, L.A. & Russell, V.A. (2005) The control of responsiveness in ADHD by catecholamines: evidence for dopaminergic, noradrenergic and interactive roles. *Dev Sci* **8**(2), 122-31.

Popescu, G. (2005) Mechanism-based targeting of NMDA receptor functions. *Cell Mol Life Sci* **62**(18), 2100-11.

Prado, M.A., Reis, R.A., Prado, V.F., de Mello, M.C., Gomez, M.V. & de Mello, F.G. (2002) Regulation of acetylcholine synthesis and storage. *Neurochem Int* **41**(5), 291-9.

Roghani, A., Feldman, J., Kohan, S.A., Shirzadi, A., Gundersen, C.B., Brecha, N. & Edwards, R.H. (1994) Molecular cloning of a putative vesicular transporter for acetylcholine. *Proc Natl Acad Sci U S A* **91**(22), 10620-4.

Rosahl, T.W., Spillane, D., Missler, M., Herz, J., Selig, D.K., Wolff, J.R., Hammer, R.E., Malenka, R.C. & Sudhof, T.C. (1995) Essential functions of synapsins I and II in synaptic vesicle regulation. *Nature* **375**(6531), 488-93.

Russell, V., de Villiers, A., Sagvolden, T., Lamm, M. & Taljaard, J. (1998) Differences between electrically-, ritalin- and D-amphetamine-stimulated release of [3H]dopamine from brain slices suggest impaired vesicular storage of dopamine in an animal model of Attention-Deficit Hyperactivity Disorder. *Behav Brain Res* **94**(1), 163-71.

Russell, V.A. (2003) Dopamine hypofunction possibly results from a defect in glutamate-stimulated release of dopamine in the nucleus accumbens shell of a rat model for attention deficit hyperactivity disorder--the spontaneously hypertensive rat. *Neurosci Biobehav Rev* **27**(7), 671-82.

Sagne, C., El Mestikawy, S., Isambert, M.F., Hamon, M., Henry, J.P., Giros, B. & Gasnier, B. (1997) Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. *FEBS Lett* **417**(2), 177-83.

Sagvolden, T. (2000) Behavioral validation of the spontaneously hypertensive rat (SHR) as an animal model of attention-deficit/hyperactivity disorder (AD/HD). *Neurosci Biobehav Rev* **24**(1), 31-9.

Sagvolden, T., Hendley, E.D. & Knardahl, S. (1992a) Behavior of hypertensive and hyperactive rat strains: hyperactivity is not unitarily determined. *Physiol Behav* **52**(1), 49-57.

Sagvolden, T., Johansen, E.B., Aase, H. & Russell, V.A. (2005) A dynamic developmental theory of attention-deficit/hyperactivity disorder (ADHD) predominantly hyperactive/impulsive and combined subtypes. *Behav Brain Sci* **28**(3), 397-419; discussion 419-68.

Sagvolden, T., Metzger, M.A., Schiorbeck, H.K., Rugland, A.L., Spinnangr, I. & Sagvolden, G. (1992b) The spontaneously hypertensive rat (SHR) as an animal model of childhood hyperactivity (ADHD): changed reactivity to reinforcers and to psychomotor stimulants. *Behav Neural Biol* **58**(2), 103-12.

Sagvolden, T. & Sergeant, J.A. (1998) Attention deficit/hyperactivity disorder--from brain dysfunctions to behaviour. *Behav Brain Res* **94**(1), 1-10.

Salinovich, O. & Montelaro, R.C. (1986) Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Anal Biochem* **156**(2), 341-7.

Siegel G.J., Agranoff Bernard W., Albers R. Wayne, Fisher Stephen K. & Uhler Michael D. (1999) Basic Neurochemistry; Molecular, cellular and medical aspects. (6), 14-15.

Soghomonian, J.J. & Martin, D.L. (1998) Two isoforms of glutamate decarboxylase: why? *Trends Pharmacol Sci* **19**(12), 500-5.

Stephenson, F.A. (2001) Subunit characterization of NMDA receptors. *Curr Drug Targets* **2**(3), 233-9.

Sudhof, T.C. (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**(6533), 645-53.

Sudhof, T.C. (2004) The synaptic vesicle cycle. *Annu Rev Neurosci* **27**, 509-47.

Sudhof, T.C. & Jahn, R. (1991) Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* **6**(5), 665-77.

Takei, Y., Harada, A., Takeda, S., Kobayashi, K., Terada, S., Noda, T., Takahashi, T. & Hirokawa, N. (1995) Synapsin I deficiency results in the structural change in the presynaptic terminals in the murine nervous system. *J Cell Biol* **131**(6 Pt 2), 1789-800.

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**(9), 4350-4.

Valtorta, F., Benfenati, F. & Greengard, P. (1992) Structure and function of the synapsins. *J Biol Chem* **267**(11), 7195-8.

van der Neut, R. (1997) Targeted gene disruption: applications in neurobiology. *J Neurosci Methods* **71**(1), 19-27.

Varoqui, H., Schafer, M.K., Zhu, H., Weihe, E. & Erickson, J.D. (2002) Identification of the differentiation-associated Na+/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J Neurosci* **22**(1), 142-55.

von Euler, G. (1991) Biochemical characterization of the intramembrane interaction between neurotensin and dopamine D2 receptors in the rat brain. *Brain Res* **561**(1), 93-8.

Walaas, S.I., Browning, M.D. & Greengard, P. (1988) Synapsin Ia, synapsin Ib, protein IIIa, and protein IIIb, four related synaptic vesicle-associated phosphoproteins, share regional and cellular localization in rat brain. *J Neurochem* **51**(4), 1214-20.

Watanabe, M., Tsuruta, S., Inoue, Y., Kinuya, M., Ogawa, K., Mamiya, G. & Tatsunuma, T. (1989) Dopamine D1 and D2 receptors in spontaneously hypertensive rat brain striatum. *Can J Physiol Pharmacol* **67**(12), 1596-7.

Yang-Feng, T.L., DeGennaro, L.J. & Francke, U. (1986) Genes for synapsin I, a neuronal phosphoprotein, map to conserved regions of human and murine X chromosomes. *Proc Natl Acad Sci U S A* **83**(22), 8679-83.

APPENDIX

I. CHEMICALS AND REAGENTS

Chemical/Reagent	**Abbreviation**	**Manifacturer**	**Cat.no**
[N-methyl-^{3}H]SCH 23390	[^{3}H]SCH 23390	Amersham Biosciences	TRK876-100UCl
DL-Dithiothreitol	DTT	Sigma Aldrich	D0632
30% Acrylamide/ Bis solution, 37.5:1	Polyacrylamide	Bio-Rad Laboratories	161-0158
Amersham ECL plus ™ Western Blotting detection reagent	ECL-plus	GE Healthcare	RPN2132
Ammonium persulfate		Sigma Aldrich	A-9164
BCA Protein Assay Kit		Pierce	23225
Bromophenol Blue	BPB	Sigma Aldrich	B5525
dH_20		-	-
Glycerol		Chemi-teknik	
Glycine		Sigma Aldrich	G7126
HRP-labelled anti-goat secondary antibody		Santa Cruz Biotechnology	Sc-2438
HRP-labelled anti-mouse secondary antibody		GE-Healthcare	NA 931
HRP-labelled anti-rabbit secondary antibody		GE-Healthcare	NA 934
Hydrochloric acid, fuming 37%	HCl	Sigma Aldrich	84426
Ketanserine tartrate		Tocris Bioscience	0908
Methanol		VWR	20 903.368
N,*N*,*N*′,*N*′-Tetramethylethylenediamine	TEMED	Sigma Aldrich	T22500
Polyethylenimine		Sigma Aldrich	468533
Ponceau S			
Potassium chloride	KCL	Merck	1.04936.1000
Powdered skimmed milk		Nestle´	
Precision Plus Protein Dual Color Standards	Protein Standard	Bio-Rad Laboratories	161-0374
Primary antibodies against		Synaptic Systems	vGLUT-2-135402

vGLUT-2 (rabbit polyclonal) vGLUT-3 (rabbit polyclonal), vACTH (rabbit polyclonal), NMDA-1 (mouse monoclonal) vGAT (mouse monoclona)		(SYSY)	vGLUT-3-135203 vACTH- 139103 NMDA-1-114001 vGAT- 131 011
Primary antibodies against COMT (mouse polyclonal), GAD65(mouse polyclonal)		BD Transduction Laboratories	COMT- 611970 GAD65- 559931
Primary antibody against Calcyon (goat polyclonal)		Santa Cruz Biotechnology	Sc-10837
Primary antibody against vGLUT-1		Made in oulaboratorie as described by Bogen et.al., 2006	
Primary antibody against synaptofysin		Sigma Aldrich	S5768
SCH23390 hydrochloride	SCH23390. HCl	Tocris Bioscience	0925
Sodium chloride	NaCl	Sigma Aldrich	S9625
Sodium dodecyl sulphate	SDS	Sigma Aldrich	L-3771
Sucrose		Sigma Aldrich	S0389
Tris (hydroxymethyl)	Tris	Sigma Aldrich	T-1378
Tween-20		Sigma Aldrich	P1379

II. CONTENT OF BUFFERS AND SOLUTIONS

a) BUFFERS AND SOLUTIONS FOR WESTERN BLOTTING

Reagents	**Ingredients**	**Amount**
Solution A	Tris-HCl (pH 8,8) SDS dH_20	45,5 g 1,0 g ad 250 ml
Solution E	Tris-HCl (pH 6,8) SDS dH_20	15,1 g 1,0 g ad 250 ml
4 x sample buffer	DTT Tris Bromophenol blue 20 % SDS 87% Glycerol dH_20	0,308 g 0,121 g 0,050 g 2,0 ml 2,3 ml 0,221 ml
Running buffer (10 X)	Glycine Tris SDS dH_20 (Running buffer 1X: 500 ml running buffer (10X) + 4,5 L dH_20	144,0 g 30,3 g 10,0 g ad 1,0 L
Towbin (10 X)	Tris Glycine dH_20 (Towbin 1 X: 500ml of Towbin 10x + 1000ml methanol+3,5L dH_20)	30,3 g 144,0 g ad 1 L
50 mM Tris-HCl buffer	Tris	12,1 g

(pH 7,4)	dH_20 Dissolve in 1,8L of dH_20, adjust to desired pH with concentrated HCl and add dH20 to 2L.	ad 2,0 L
Blocking buffer (5%)	Powdered skimmed milk 5 % Azid (NaN_3) TBS-Tween	2,5 g 100 µl ad 50 ml
Ponceau S	Ponceau Acetic acid (100%) dH_20	2 g 30 ml ad 1 L
ECL-plus	**Assuming 1 nitrocellulose filter:** Solution A Solution B	 2 ml 50µl

b) BUFFERS AND SOLUTIONS FOR RECEPTOR BINDING ASSAY

Reagents	**Ingredients**	**Amount**
50 mM Tris-HCl buffer **(pH 7,4)**	Tris dH_20 Dissolve in 1,8L of dH_20, adjust to desired pH with concentrated HCl and add dH20 to 2L.	12,1 g ad 2,0 L
50mM Tris-HCL incubation buffer (pH 7.4)	Tris NaCl KCl $CaCl_2$ Mg Cl_2 dH_20 Dissolve in 1,8L of dH20, adjust to desired pH with. concentrated HCl and add dH20 to 2L	12,1 g 14,0 g 0,75 g 0,59 g 0,19g ad 2 L

c) SOLUTIONS FOR PREPARATION OF DIAPHRAGM AND TONGUE

Reagents	Ingredients	Amount
1M KCl	KCl dH_20	3,78 g ad 50 ml
10 % TCA/acetone	TCA Acetone	1 ml 9ml
Urea Sample buffer	Tris/glycine (10X stock), pH 8.6 DTT Urea dH_20 (Tris/glycine (10 % stock) made of 2,4 g Tris+ 1,6g glycine in 100 ml dH_20 . Adjusted to desired pH with concentrated HCl	1ml 15,4 mg 4,8 mg Ad 10 mL
Acetone/DTT/NaF	DTT NaF Acetone	77,1 mg 42 mg Ad 50 ml

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